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INTRODUCTION

The major histocompatibility complex (MHC) class I genes encode proteins which are chiefly involved in presentation of intracellularly-derived peptides to cytotoxic T cells. Thereby they play a key role in the initiation of an immune response (Klein 1986, Cresswel 1994). The MHC class I and class II molecules are structurally strikingly similar, and they are both expressed on the surface of antigen-presenting cells as heterodimeric molecules. Class I molecules are composed of α light chain, β2 microglobulin (β2m), and a heavy α chain. The class II molecule is similarly composed of an α and β chain. The MHC subunits are all encoded in the MHC region except β2m which is encoded by *A* gene on a separate chromosome in all mammals investigated.

The MHC is one of the most well-studied gene regions among vertebrate species. A major impetus for this strong interest is that some MHC genes and gene products exhibit a remarkably high level of genetic polymorphism. Moreover, MHC is particularly interesting since it is one of the few polymorphic systems where it has been possible to firmly establish a functional significance for the observed genetic variation. Thus, the MHC has been intensively studied in the fields of immunology, genetics, and evolutionary biology. The available data on MHC structure and function strongly imply that the MHC diversity has evolved as response to the selection pressure on the vertebrate immune system to recognize and eliminate invading parasites and microorganisms. Genetic diversity of MHC molecules is generated by two mechanisms, gene duplications and allelic polymorphism. Both peptide-presenting class I and class II molecules are encoded by multiple loci. The presence of two or more linked loci, which encode distinct molecules, are

denoted "fixed heterozygosity", implying that even homozygotes possess multiple forms of antigen-presenting molecules.

The history of research on *Bos taurus* and *Bos indicus* ("bovine") major histocompatibility complex (MHC) cannot be understood without a general appreciation for the mouse *H-2* complex and human leukocyte antigen (*HLA*) system. Like *HLA*, research on the bovine MHC has deep roots in classical serology and transplantation genetics. The discovery of MHC of cattle is attributed to Amorena and Stone (1978), and Spooner and co-workers (1978). The genetic region they and others defined using serological reagents produced by skin transplants and alloimunization was named the bovine lymphocyte antigen (*BoLA*) system (Spooner *et al.* 1979). Workers in the field unanimously agreed to retain the name *BoLA* rather than *Bota* (Klein *et al.* 1991) because of the long and rich history of research conducted on MHC of cattle, and to avoid unnecessary confusion. Among all species studied, the *BoLA* system ranks next in term of depth of knowledge compared with *HLA* and *H-2*.

Expression of the classical class II heterodimers on cattle leukocytes was identified using cross-species-reactive monoclonal antibody (mAb) (Lewin *et al.* 1985a). The identification of mAb with apparent locus-specificity was first recognized on B cells from subset of cows infected with bovine leukemia virus (BLV) (Lewin *et al.* 1985b). These mAb were later confirmed to be a specific for *DR* and *DQ* products by sequential immunoprecipitation and 1D-IEF (Janzer-Pfeil and Splitter 1989; Bissumbher *et al.* 1994). While it is clear that distinct *DR* and *DQ* products are expressed on the cell surface, *DY, DI*, or *DO* products have not been identified.

Cytoplasmic and cell-surface expression of class II antigens has been studied using immunofluorescence (Lewin *et al.* 1985a), antibody-dependent complement-mediated cytotoxicity

(Lewin *et al.* 1985b), flow cytometry (Lewin *et al.* 1985b; Davis *et al.* 1987), immunoprecipitation (Hoang-Xuan *et al.* 1982), and 1D-IEF (Joosten *et al.* 1989). Expression has been demonstrated on B cells (Lewin *et al.* 1985b), activated T cells (Lalor *et al.* 1986), cell lines (Ababou *et al.* 1994) alveolar macrophages (Ohmann *et al.* 1986), monocytes (Taylor *et al.* 1993; Hughes *et al.* 1994), and mammary (Fitzpartrick *et al.* 1992) and bronchial (Spurzem *et al.* 1992) epithelial cells. In contrast to the *HLA-DR* and *–DQ* products, which predicted the existence of discrete loci on the basis of serological and biochemical data, *BoLA-DR* and *–DQ* genes were detected first (Andersson *et al.* 1986a; 1986b). Expression of class II genes has been studied at the transcriptional level using Northern blot analysis (Burke *et al.* 1991; Stone and Muggli-Cockett 1993), cDNA cloning (Xu *et al.* 1991: 1993: 1994), and PCR-RFLP (Xu *et al.* 1994). The class IIa region of cattle contains the known express class II genes: *DRA, DRB3, DQA1, DQA2, DQB1,* and *DQB2* (Aida *et al.* 1995; Nishino *et al.* 1995; Dikiniene and Aida 1995; Aida 1995) . In at least three haplotypes with duplicated *DQ* genes, both *DQB* genes are expressed (Xu *et al.* 1994; Aida 1995). Expression of other genes in the class IIa region, *DRB1* (a pseudogene), *DRB2*, *DQA2*, and the class IIb region genes, *DYA, DYB, DIB*, and *DOB*, has not been demonstrated to date. Additionally, comparison transcription, expression and molecules ability of antigen presenting of DR and DQ reveal that BoLA-DR molecule had the highest immune response ability in class II molecules (Aida 1995). So that, to study of *BoLA-DRB3* is the most important for study of the association with BoLA class II and diseases.

In this study, we describe the development of polymerase chain reaction-sequences based typing (PCR-SBT) which can assign *BoLA-DRB3* alleles of large numbers of animals and test this method on Japanese Shorthorn cattle in experimental farm, graduate school of agricultural science,

Tohoku university (Kawatabi farm). And then, we calculate the *BoLA-DRB3*allele frequencies in four cattle breeds such as Holstein, Jersey, Japanese Shorthorn and Japanese Black that the essential data for study of disease association. Last, to investigated the relationship between polymorphism of *BoLA* gene and resistance or susceptibility to BLV-induced lymphoma, the nucleotide sequencing of *exon 2* of the *BoLA-DRB3* gene of 81 BLV-infected and of control Japanese Black cattle was determined by PCR-sequenced-based typing.

CHAPTER 1

Identification of new bovine *BoLA-DRB3* alleles by sequence-based typing

Abstract

The products of the *BoLA-DRB3* locus are important in the bovine immune response. In this study, we developed a new method for sequence based typing (SBT) of alleles of this locus that appears to be generally applicable to all *BoLA* class II genes. First, we performed an initial round of amplification by PCR using conserved locus-specific primers. Then we performed PCR with individual sequence-specific primers (SSPs). For this second round of amplification, we used a locus-specific primer and at least one group-specific primers to amplify all the alleles that belonged to eight groups. These eight groups of alleles were categorized in terms of the sequence of amino acids 9-13 in the first hypervariable region of BoLA-DRβ. The locus-specific primer DRB3ALL was also used for the second PCR to avoid to skipping a new allele or one of the recognized alleles. Next, we performed cycle sequencing of each product using an M13-tailed system. Using our method, we investigated 53 animals whose *BoLA-DRB3* haplotypes had been characterized at the Fifth International BoLA Workshop. I identified 27 different alleles that included four new alleles in 43 different heterozygous and two different homozygous combinations. The reproducibility was 100% for all samples on two separate occasions. The results of PCR-SBT exactly matched the PCR-RFLP patterns as defined at the Fifth International BoLA Workshop. Collectively, the

results suggest that our method allows detection of as yet undefined alleles that consist of new combinations of known sequences.

Introduction

The major histocompatibility complex (MHC) of cattle is known as *BoLA* and is located on chromosome 23 (BTA23). Physical mapping of BTA23 demonstrated that the MHC class I region encompasses approximately 1,550 kilobase pairs (kbp) of DNA, and that there are two tightly linked loci that are expressed (*BoLA-A* and *BoLA-B*) (Bensaid *et al.* 1991). The MHC class III region consists of a heterogeneous set of genes that are related to immunological and other functions, such as the genes for complement factors BF and C4, steroid 21-hydroxylase (*CYP21*), heat shock protein 70 (*HSP71*) and tumor necrosis factors α and β (*TNFA* and *TNFB*) (Lewin *et al.* 1996). One of the notable differences between the genomic organization of the MHCs of the bovine and those of the human and the mouse is the splitting of the MHC class II region into two subregions that are separated by at least 15 cM (centiMorgans) (van Ejik *et al.* 1995; Anderson *et al.* 1988). The class IIa subregion consista of two clusters of genes, *DR* and *DQ*, which lie in close proximity (Andersson *et al.* 1986a). The class IIb region includes the *DMA*, *DMB*, *LMP2*. *LMP7* and *TAP* genes (Davis *et al.* 1997; Russell *et al.* 1997; Band *et al.* 1998), which are involved in the processing and transport of antigens, and other class II–like genes, such as *DNA*, *DOB*, *DIB*, *DYA* and *DYB*, whose functions are unknown (Lewin *et al.* 1996). The *DR* region consists of one *DRA* locus and at least three *DRB* loci, with *exon 2* of the *DRB3* gene being highly polymorphic (Russell

et al. 1997). To data, more than 63 *DRB3* alleles have been identified by DNA sequencing of *exon 2* (Davies *et al.* 1997; Russell *et al.* 1997).

Successful *BoLA-DRB3* typing has been achieved by use of a number of methods individually or in combination (Davies *et al.* 1992), including serology (Davies and Antczak 1991b), mixed lymphocyte culture (Davies and Antczak 1991a), isoelectric focusing (IEF) (Watkins *et al.* 1989; Glass *et al.* 1992), restriction fragment length polymorphism (RFLP) analysis (Andersson *et al.* 1986a), PCR-RFLP (van Eijk *et al.* 1992), T cell typing with cell lines and clones (Rothel *et al.* 1990), heteroduplex analysis (Sitte *et al.* 1995), sequence-specific oligonucleotide (SSO) typing (Sitte *et al.* 1996), denaturing gradient gel electrophoresis (DGGE) (Aldrige *et al.* 1998), analysis of a microsatellite adjacent to *DRB3* (Ammer *et al.* 1992; Ellegren *et al.* 1993), and sequencing of genomic DNA, cDNA or cloned PCR products (Aida *et al.* 1995; Burke *et al.* 1991; Muggli-Cockett and Stone 1989; Groenen *et al.* 1990; Sigurdardottir *et al.* 1991, Xu *et al.* 1993b). Most of these methods are labour-intensive and their ability to define new allele is limited, a severe drawback in studies of polymorphic loci. More precise sequence-based typing (SBT), as developed for the human MHC (HLA) (McGinnis *et al.* 1995; Spurkland *et al.* 1993; Knipper *et al.* 1994; Santamaria *et al.* 1992), would improve the resolution and the accuracy of typing of MHC alleles in animals.

In this study, we describe the development of a method for PCR-SBT which allows identification of the *BoLA-DRB3* alleles of large numbers of animals with relative ease, and we discuss the relationship between the results of PCR-SBT typing and the identification of *BoLA-DRB3* alleles by PCR-RFLP analysis.

Materials and Methods

Animals and extraction of DNA

Fifty-three individuals of eight cattle breeds (Ayrshire, British Friesian, Danish Black Pied, Danish Red, Jersey, Holstein-Friesian, Limousin and Hereford) were used in this study. These animals were typed for BoLA-A antigens by serology and IEF (Davies *et al.* 1994a), and for *BoLA-DQA*, *-DQB*, *-DYA*, *-DOB*, *-DIB* and *-DRB* by serology, IEF, RFLP analysis and PCR-RFLP analysis (Davies *et al.* 1994b) as part of the Fifth International BoLA Workshop.

Genomic DNA was extracted from whole blood with 10% SDS and phenol-chloroform (McKnight, 1978) and dissolved in distilled water of 20 ng/μl.

Amplification of BoLA-DRB3 exon 2 by PCR

All the oligonucleotide primers for amplification of *exon 2* of *BoLA-DRB* are shown in Table 1. For the first round the PCR, we prepared the locus-specific primers ERB3N and HL031 (van Eijk *et al.* 1992) that included nine and eight nucleotides at the 5' and 3' ends of *exon 2*, respectively, plus intron sequences, were prepared. Next, we performed PCR-sequence-specific primer (SSP) amplification using distinct group-specific 3' primers (Sp1 through Sp8) and the locus-specific 5' primer DRB3B. The sequences of the SSPs for the selective amplification of the eight groups of alleles were based on previously published sequences of *BoLA-DRB3* alleles, as shown in Fig. 1-1. The *BoLA-DRB3* alleles were divided into eight groups (G1 through G8) by reference to the sequence of amino acids 9-13 in first hypervariable region of the BoLA-DRβ chain. The oligonucleotide primers for amplification all alleles in each group were constructed and

designated Sp1 through Sp8. In addition, a locus-specific 3' primer, DRB3ALL, was designed for amplification of the alleles in all eight groups. DRB3B consisted entirely of nucleotides at the 3' end of *exon 2* and had a six-bp overlap with the 3' end of HL031.

Reactions were performed with 20 ng of DNA (1 μl) in a 50 μl (final volume) of a reaction mixture that contained 49 μl of PCR buffer [final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% TritonX-100], with 120 μM dNTPs, 1.5 mM $MgCl_2$, 200 μM each primer and 1 unit of recombinant Taq DNA polymerase (rTaq) (TOYOBO). The thermal cycling profile for the first round of amplification was as follows: initial denaturation for 5 min at 95℃, followed by 20 cycles of 50 sec at 95℃, 50 sec at 60℃ and 50 sec at 72℃, with final extension for 2 min at 72℃. Subsequently, 1 μl of the reaction mixture after the first-round PCR was transferred to well of a 96-well PCR plate. Each well contained 24 μl GeneAmp^R Gold Buffer [final concentrations: 50mM KCl, 10mM Tris-HCl (pH8.3), 0.1% TritonX-100] with 120 μM dNTPs, 1.5 mM $MgCl_2$, 400 μM each primer and 1 unit of Taq polymerase (AmpliTaq^{TM} Gold) for PCR-SSP with the following thermal cycling profile: initial denaturation for 10 min at 95℃, followed by 20 cycles of 1 min at 95℃, 30 sec at 64℃ and 30 sec at 72℃, with final extension for 5 min at 72℃. All PCRs were performed in a Tgradient Thermocycler 96 (Biometra biomedizinische Analytik GmbH, Goettingen, Germany).

Five μl of the reaction mixture after PCR-SSP were fractionated by electrophoresis on a 2% agarose/TAE gel. The gel was stained with ethidium bromide and bands of DNA were visualized under UV light.

Identification of alleles by direct sequencing

I used a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, Conn) and a 5 x Sequencing Buffer (PE Applied Biosystems) to sequence each product of PCR-SSP with the -21M13 primer 5'-TGT AAA ACG ACG GCC AGT-3' or with the M13Reverse primer 5'-CAG GAA ACA GCT ATG ACC-3'. One µl of the reaction mixture after the second round of PCR, 3 pmol of sequencing primer, 1 µl of Terminator Ready Reaction Mix, 1.5 µl of half BD and 5.5 µl of distilled water were combined, and cycle sequence reactions were performed in accordance with the conditions in the manual from PE Applied Biosystems. Reaction mixtures were then subjected to automated loaded analysis with an ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems). Data were collected by computer with the software package provided with the sequencing system.

Analysis of data

After the collection of raw data, results of chromatography were analyzed with the Sequencing Analysis™ and Factura™ (PE Applied Biosystems). Nucleotide sequences were analyzed using the BLAST (Gish and States 1993) algorithm to search the GenEMBL databases.

Results

Classification of eight groups of alleles by comparison of known BoLA-DRB3 alleles and construction of group-specific primers

An alignment of the predicted amino acid sequences of the β1 domains encoded by 88 previously characterized *BoLA-DRB3* alleles are shown in Fig. 1-1. In all, 32 polymorphic amino acid residues were identified in these alleles and most of the variability was concentrated in four hypervariable regions, namely, HV1 (codons 9-13), HV2 (codons 26-38), HV3 (codons 56-61) and HV4 (codons 66-78), which are involved in the recognition of antigens and T cell receptors (TCR) and which were recognized for the first time in this study. The sequences could be divided into eight groups of alleles, G1 through G8, according to the amino acids sequence in the first hypervariable region HV1, namely, codons 9 through 13. Therefore, for subtyping of the *BoLA-DRB3* alleles in these eight groups, I designed group-specific primer that showed anneal to seven to eleven nucleotides in *exon 2*, which encodes the first hypervariable region HV1 (Table 1 and Fig. 2B). Moreover, all of the primers included nucleotides at the 5' end of ERB3N (a locus-specific primer) and nucleotides of a -21M13 primer.

Subtyping of BoLA-DRB3 alleles by PCR-SSP using group-specific primers

To specifically and similarly amplify *exon 2* of *BoLA-DRB3* alleles that belonged to the eight groups of alleles from heterozygous or homozygous animals, we performed the first round of amplification by PCR using the locus-specific primers ERB3N and HL031 (Fig. 1-2A). For the subsequent amplification by PCR-SSP, we used at least one of the group-specific primers Sp1

through Sp8 and the locus-specific primer DRB3B (Fig. 1-2B). In addition to the group-specific primers, to avoid to missing a new allele or one of the recognized alleles, we also used the locus-specific primer DRB3ALL for second rounds of amplification (Fig.1- 2B and Table 1-1).

The 52 animals that had been defined in terms of *BoLA-DRB3* haplotype by PCR-RFLP typing at the Fifth International BoLA Workshop were used to establish the unambiguous discrimination by our method of the groups of *BoLA-DRB3* alleles (Table 1-2). Using the combinations of primers summarized in Table 1, we were able, in all cases, clearly to distinguish the eight independent groups of *BoLA-DRB3* alleles by amplification in eight second-round sets of PCRs. Since *exon 2* of *BoLA-DRB3* in 43 (83%) of the 52 animals was amplified as two separate products of PCR (Fig. 1-3A) with 18 different combinations of groups, these cases were considered to represent heterozygotes. By contrast, the remaining nine individuals, namely, WK5-11, WK5-16, WK5-19, WK5-22, WK5-33, WK5-34, WK5-42, WK5-45 and WK5-56, each generated only a single product (Figs. 1-4A to1-6A). In addition with all samples, the second amplification step produced an obvious product when locus-specific primer pair DRB3ALL and DRB3B was used (Figs. 1-3A to 6A). All samples of DNA were analyzed twice and the reproducibility of the results of PCR-SSP was 100% in all animals. No false-positive or false-negative results of typing were obtained.

Sequencing of products of PCR-SSP and assignment of allele

After PCR-SSP typing of *BoLA-DRB3* alleles, the amplified products obtained were used for subsequent one-directional cycle sequencing with a -21M13 primer (Fig. 1-2C).

In the cases of 43 animals that had been shown to be heterozygous by PCR-SSP, the sequencing chromatogram obtained from one product of PCR of a single allele was interpreted automatically by the computer software and identification of the *BoLA-DRB3* allele was then made by comparison with the sequences of all known *BoLA-DRB3* alleles. Fig. 1-3 shows the results of DNA amplification and sequencing chromatograms of two products of PCR from a heterozygous animal (WK5-38), whose alleles belonged to groups G1 and G2. Heterozygosity in such cases was, thus, confirmed by direct sequencing of products of PCR-SSP from alleles in different groups (Table 1-2).

I next examined the nine animals that each generated only one product of PCR. These animal were expected to be homozygous or, alternatively, heterozygous for alleles in the same group. In these cases, to avoid to failure the amplify a new allele, we used the product of PCR that had been amplified with the ALL and DRB3B primers for direct sequencing. Only two animals, WK5-16 and WK5-45, each yielded a single sequence (homozygosity) (Fig. 1-4), while five animals, namely, WK5-11, WK5-33, WK5-34, WK5-42 and WK5-56, yielded a typical heterozygous pattern (Fig. 1-5). By contrast to results for homozygous animals, as shown in Fig. 1-5B, at the heterozygous positions 254-258 (codons 84 and 85) there were two peak, each of approximately half the height of peaks associated with homozygosity. Although, in these cases, it was difficult to assign heterozygosity accurately and reliably in the terms of polymorphic positions, a comparison with the sequences of all known *BoLA-DRB3* alleles which were included in allelic group specificies determined by PCR-SSP typing allowed identification of polymorphic residues. Moreover, animal WK5-19, which was heterozygous for the *DRB3*0201* allele with a 3-bp deletion at nucleotide positions 193-195 (corresponding to codon 65), gave an unreadable result

(Fig. 6B). Therefore, to obtain good sequence data, the C-terminal 195-282 bp of this fragment was sequenced with an M13Reverse primer (Fig. 1-6C). Similarly, two other alleles, *DRB3*3301* and **4101*, appeared to have an amino acid deletion at codon 65 (Fig. 1-1).

Thus, using PCR-SBT, we identified 27 different *BoLA-DRB3* alleles which included four newly identified alleles, in 43 different heterozygous and two different homozygous combinations (Table 1-2 and Fig. 1-7). The PCR-RFLP patterns predicted from the DNA sequences determined by PCR-SBT exactly matched the patterns as defined at the Fifth International BoLA Workshop. In addition, the concordance between results of analyses by PCR-SBT and PCR-SSP was 100% for all in 53 animals.

Identification of new alleles by PCR-SBT

Fig. 1-8 summarizes the four new alleles found in this study. The new *BoLA-DRB3* sequences were named by the BoLA Nomenclature Committee of the International Society for Animal Genetics (http://www2.ri.bbsrc.ac.uk/bola). One new allele, similar to *DRB3*2001*, designated *DRB3*2006*, differed from *DRB3*2001* at positions 38 and 140. An other new allele was designated *DRB3*2502* and differed from *DRB3*25012* at positions 167, 170, 220 and 222. *DRB3*4501* was an allele with greatest sequence similarity to the *DRB3*46011* (97.4% identity at the nucleotide level). The remaining *DRB3*4401* allele differed from *DRB3*3301* at positions 32, 199, 200, 209, 210, 211, and 213 (97.0% identity at the nucleotide level). In addition, these alleles were 90.3% to 94.9% identical at the nucleotide level to *BoLA-DRB3* cDNA clone NR-1 (87.2% to 94.8% homology at the amino acid level) (Fig. 1-8). Although ours was not a random sample of cattle, we examined the distribution of *DRB3* alleles in the various breeds of cattle, as shown in

Table 1-3. All four new alleles were found in Jersey cattle and not in other breeds. Thus, our results suggest that our new version of PCR-SBT allows detection of as yet undefined alleles that are composed of new combinations of known sequences.

Discussion

There are various methods for the DNA-based typing but PCR-SBT provides a direct and high-resolution method for the identification of genetic polymorphisms (Santamaria *et al.* 1992; Spurkland *et al.* 1993; Knipper *et al.* 1994; Voorter *et al.* 1997; Scheltinga *et al.* 1998; Kotsch *et al.* 1999). In this study we tested the applicability of high-resolution PCR-SBT to the *BoLA-DRB3* locus by typing a panel of 52 samples that had been characterized in terms of *BoLA-DRB3* haplotypes at the Fifth International BoLA Workshop. The first amplification step, with a single locus-specific primer pair, generated a product of about 306 bp. This step allowed us to decrease the amount of starting material needed and to unify the concentrations of templates for subsequent PCR. In addition, the uniformity in the amount of each template for the second-round PCR should eliminate false-positive and false-negative results. The second round of PCR used a mixture of primers that consisted of a locus-specific primer and at least one group-specific primer that could allow amplification of all alleles in one of eight groups of alleles. Finally, cycle sequencing of each product of PCR-SSP was performed using an M13-tailed system. One potential drawback of our method of PCR-SBT, is that it does not provide the entire sequence of *exon 2*. It relies on PCR-based generation of each template using amplification primers located at

the 5' end of *exon 2* encoding the first hypervariable region of BoLA-DRβ. Consequently, potential variations in sequence in these regions might remain unexamined and new alleles might be overlooked. Therefore, to resolve this problem, we also prepared a locus-specific primer, DRB3ALL, for amplification of all *BoLA-DRB3* alleles for the second-round PCR. Here, we successfully typed a total of 52 samples using PCR-SBT and identified 27 different alleles, including four new alleles, in 43 different heterozygous and two different homozygous combinations. The concordance between the results of the method using PCR-SBT and the results of PCR-RFLP typing or PCR-SSP typing was 100%. Moreover, our approach allowed high-resolution *BoLA-DRB3* typing of a series of the ten samples of DNA in 24 h, from the initial amplification to the final identification of each allele. Our rapid and accurate procedure showed be extremely useful in view of the potential clinical applications of high-volume *BoLA-DRB3* typing.

In the present study, we identified four new alleles in the 52 animals that had been characterized at the Fifth International BoLA Workshop. These new alleles were restricted to the Jersey breed, as follows: in WK5-56, *DRB3*4401*; in WK5-17, WK5-18, WK5-56 and WK5-61, *DRB3*4501*; in WK5-61, *DRB3*2502*; and in WK5-58, *DRB3*2006*. Our results confirm the data presented by Gilliespie *et al.* (1999) that *DRB3.2*10* and *DRB3.2*15* are the most frequent alleles in Jersey cows: the PCR-RFLP types *DRB3.2*10* and *DRB3.2*15* correspond to our alleles *DRB3*2006* and *DRB3*4501*, respectively. Among the 24 previously published alleles, the *DRB3*0301* allele was found in Jersey cows, exclusively. Thus, five out of seven alleles that were detected in Jersey cows were not found in a variety of other breeds, indicating that differences exist between breeds of cattle with respect to frequencies of *BoLA-DRB3* alleles. More studies of the

BoLA-DRB3 gene of Jersey cows are needed to determine the frequency of various alleles within the Jersey breed.

An ideal genome-typing technique should provide as much information as possible (Spurkland *et al.* 1993) and the quality of the results should be easy to evaluate. Sequencing is the only method that provides full information about the gene segment studied. Although cloning and sequencing of products of PCR have been used for high-resolution *BoLA-DRB3* typing (Sigurdardottir *et al.* 1991), a long time and much work are required to obtain the desired information. In addition, the reliability of sequences can be low because of errors (Sigurdardottir *et al.* 1991) and mosaic sequences, introduced by "Jumping" PCR (Paabo *et al.* 1989; Sigurdardottir *et al.* 1991) during the amplification by PCR of *BoLA-DRB3* alleles in heterozygous individuals. Therefore, our approach, using PCR-SBT, allows rapid *BoLA-DRB3* typing of cattle and has distinct advantages over many previous BoLA-typing techniques, in particular in terms of sensitivity, specificity and potential for identifying new *BoLA-DRB3* alleles. In addition, PCR-SBT is rapid, relatively easy to perform, and facilitates the sequencing of *BoLA-DRB3* alleles without cloning. The application of the MHC-typing methods described in this paper need not be restricted to examination of the BoLA class II locus since the techniques are DNA sequencing-dependent and can be universally applied to other loci (Myers *et al.* 1988; Weber *et al.* 1991; Lessa 1993). The application of PCR-SSP and direct sequencing to other BoLA loci might facilitate studies of the class I and class II genes that have been difficult to examine by the BoLA-typing techniques applied to date.

Table 1-1
Oligonucleotide primers for the first-round PCR and subsequent PCR-SSP

	Orientation	Primer	Sequence	Length (bp)	Location (nucleotide)	Tm[f] (℃)
1st PCR	sense	ERB3N	GGA ATT CCT CTC TCT GCA GCA CAT TTC C	28	intron 14 bp + exon 2 14-22	67.4
	antisense	HL031[a]	TTT AAA TTC GCG CTC ACC TCG CCG CT	26	exon 2 275-282 + intron 10 bp	75.4
2nd PCR	sense	Sp1[b,d]	tgt aaa acg acg gcc agt AGC ACA TTT CCT GCA GTA TC	38	exon 2 23-34	58.6
		Sp2[b,d]	tgt aaa acg acg gcc agt AGC ACA TTT CCT GGA GTA TTC TAA	42	exon 2 23-35	61.2
		Sp3[b,d]	tgt aaa acg acg gcc agt AGC ACA TTT CCT GGA GTA TTA	39	exon 2 23-32	57.1
		Sp4[b,d]	tgt aaa acg acg gcc agt AGC ACA TTT CCT GGA GTA TTG	39	exon 2 23-32	60.0
		Sp5[b,d]	tgt aaa acg acg gcc agt CAC ATT TCC TGG AGT ATG	36	exon 2 23-31	52.4
		Sp6[b,d]	tgt aaa acg acg gcc agt GCA CAT TTC CTG GAG TAT C	37	exon 2 23-31	56.2
		Sp7[b,d]	tgt aaa acg acg gcc agt AGC ACA TTT CCT GGA GTA TA	38	exon 2 23-31	55.5
		Sp8[b,d]	tgt aaa acg acg gcc agt CAC ATT TCC TGG AGT ATT CTA C	40	exon 2 23-35	56.1
		DRB3ALL[b,d]	tgt aaa acg acg gcc agt ATT CCT CTC TCT GCA GCA CAT TTC CTG	45	exon 2 23-24	71.7
	antisense	DRB3B[c,e]	cag gaa aca gct atg acc CGC CGC TGC ACA GTG AAA CTC	39	exon 2 261-274	72.2

[a] Primer HL031 was described by van Eijk et al. (1992).

[b] Lowercase letters denote complementary M13(-21) universal primer.

[c] Lowercase letters denote complementary M13 reverse primer.

[d] The underlined sequence overlaps with the 3' end of ERB3N.

[e] The underlined sequence overlaps with the 3' end of HL031.

[f] Tm = melting temperature

Table 1-2
Results of *BoLA-DRB3* typing of animals of the Fifth International BoLA Workshop.

Animal number of Fifth BoLA Workshop	*BoLA-DRB3* SBT analysis		PCR-SSP typing		Method used for analysis[a]	*DRB3* PCR-RFLP	
	A	B	A	B		A	B
WK5-01	*0801	*1101	G5	G1	1	21	22
WK5-02	*0901	*1501	G2	G8	1	16	11
WK5-03	*1601	*3202	G7	G4	1	10	12
WK5-04	*0201	*3201	G8	G4	1	7	12
WK5-05	*0501	*1801	G6	G5	1	1	18
WK5-06	*1001	*1101	G2	G1	1	3	22
WK5-07	*0201	*0902	G8	G2	1	7	11
WK5-08	*0101	*1102	G2	G3	1	24	22
WK5-09	*0901	*1601	G2	G7	1	11	10
WK5-10	*14011	*1601	G1	G7	1	27	10
WK5-11	*1101	*14011	G1	G1	2	22	27
WK5-12	*1101	*20012	G1	G4	1	22	15
WK5-13	*1101	*20012	G1	G4	1	22	15
WK5-14	*0902	*20012	G2	G4	1	11	15
WK5-15	*14011	*20012	G1	G4	1	27	15
WK5-16	*20012	*20012	G4	G4	2	15	15
WK5-17	*0301	*4501	G4	G3	1	9	15
WK5-18	*0201	*4501	G8	G3	1	7	15
WK5-19	*0201	*1501	G8	G8	3	7	16
WK5-20	*0201	*1201	G8	G7	1	7	8
WK5-21	*0101	*1501	G2	G8	1	24	16
WK5-22	*0201	*1501	G8	G8	3	7	16
WK5-23	*0201	*1201	G8	G7	1	7	8
WK5-24	*0201	*1201	G8	G7	1	7	8
WK5-25	*1201	*1801	G7	G5	1	8	18
WK5-26	*1102	*1501	G3	G8	1	22	16
WK5-27	*1102	*1201	G3	G7	1	22	8
WK5-28	*0701	*1501	G4	G8	1	28	16
WK5-29	*0101	*1201	G2	G7	1	24	8
WK5-30	*0101	*2002	G2	G4	1	24	15
WK5-31	*1001	*1101	G2	G1	1	3	22
WK5-32	*1001	*1801	G2	G5	1	3	18
WK5-33	*0101	*0902	G2	G2	2	24	11
WK5-34	*1002	*1102	G3	G3	2	3	22
WK5-35	*0801	*1101	G5	G1	1	21	22
WK5-36	*0701	*1102	G4	G3	1	28	22
WK5-37	*0701	*1001	G4	G2	1	28	3
WK5-38	*1001	*2801	G2	G1	1	3	31
WK5-39	*0101	*0701	G2	G4	1	24	28
WK5-40	*0201	*2801	G8	G1	1	7	31
WK5-41	*0101	*0201	G2	G8	1	24	7
WK5-42	*1101	*2801	G1	G1	2	22	31
WK5-43	*1101	*1501	G1	G8	1	22	16
WK5-44	*1501	*2801	G8	G1	1	16	31
WK5-45	*1101	*1101	G1	G1	2	22	22
WK5-46	*1101	*1801	G1	G5	1	22	18
WK5-49	*0701	*1201	G4	G7	1	28	8
WK5-51	*0701	*1201	G4	G7	1	28	8
WK5-56	*4401	*4501	G3	G3	2	20	15
WK5-58	*0801	*2006	G5	G7	1	21	10
WK5-59	*0101	*2703	G2	G3	1	24	23
WK5-61	*2502	*4501	G8	G3	1	17	15

[a]1, Two products of PCR-SSP from an animal heterozygous for alleles in different groups of alleles were sequenced.

2, One product of PCR-SSP from an animal homozygous or heterozygous for alleles belonging in the same group of alleles was sequenced.

3, One product of PCR-SSP from an animal heterozygous for an allele with a 3-bp deletion at nucleotide positions 193-195 was sequenced.

Table 1-3
Distribution by breed of *BoLA-DRB3* alleles in animals examined at the Fifth International BoLA Workshop

		Number of animals of each breed possessed the allele							
DRB3 allele	Total n=52	British Friesian n=14	Danish Black Pied n=20	Danish Red n=8	British Friesian x Ayrshire n=1	Limousin x Ayrshire n=1	British Friesian x Hereford n=2	Holstein Friesian n=1	Jersey n=5
0101	8	4	3	0	0	0	0	1	0
0201	10	1	7	0	0	0	1	0	1
0301	1	0	0	0	0	0	0	0	1
0501	1	0	0	0	0	0	1	0	0
0801	3	2	0	0	0	0	0	0	1
0901	2	0	0	1	1	0	0	0	0
0902	3	2	0	1	0	0	0	0	0
1001	5	3	2	0	0	0	0	0	0
1101	11	4	4	3	0	0	0	0	0
1201	8	3	5	0	0	0	0	0	0
1401	3	0	0	3	0	0	0	0	0
1501	8	0	7	0	1	0	0	0	0
1601	3	0	0	2	0	1	0	0	0
1801	4	1	2	0	0	0	1	0	0
20012	5	0	0	5	0	0	0	0	0
3201	1	0	0	0	0	0	1	0	0
3202	1	0	0	0	0	1	0	0	0
1102	5	3	2	0	0	0	0	0	0
0701	6	3	3	0	0	0	0	0	0
1002	1	1	0	0	0	0	0	0	0
2002	1	1	0	0	0	0	0	0	0
2801	4	0	4	0	0	0	0	0	0
2703	1	0	0	0	0	0	0	1	0
*4401**	1	0	0	0	0	0	0	0	1
*2502**	1	0	0	0	0	0	0	0	1
*4501**	4	0	0	0	0	0	0	0	4
*2006**	1	0	0	0	0	0	0	0	1

* These alleles were found for the first here in this study.

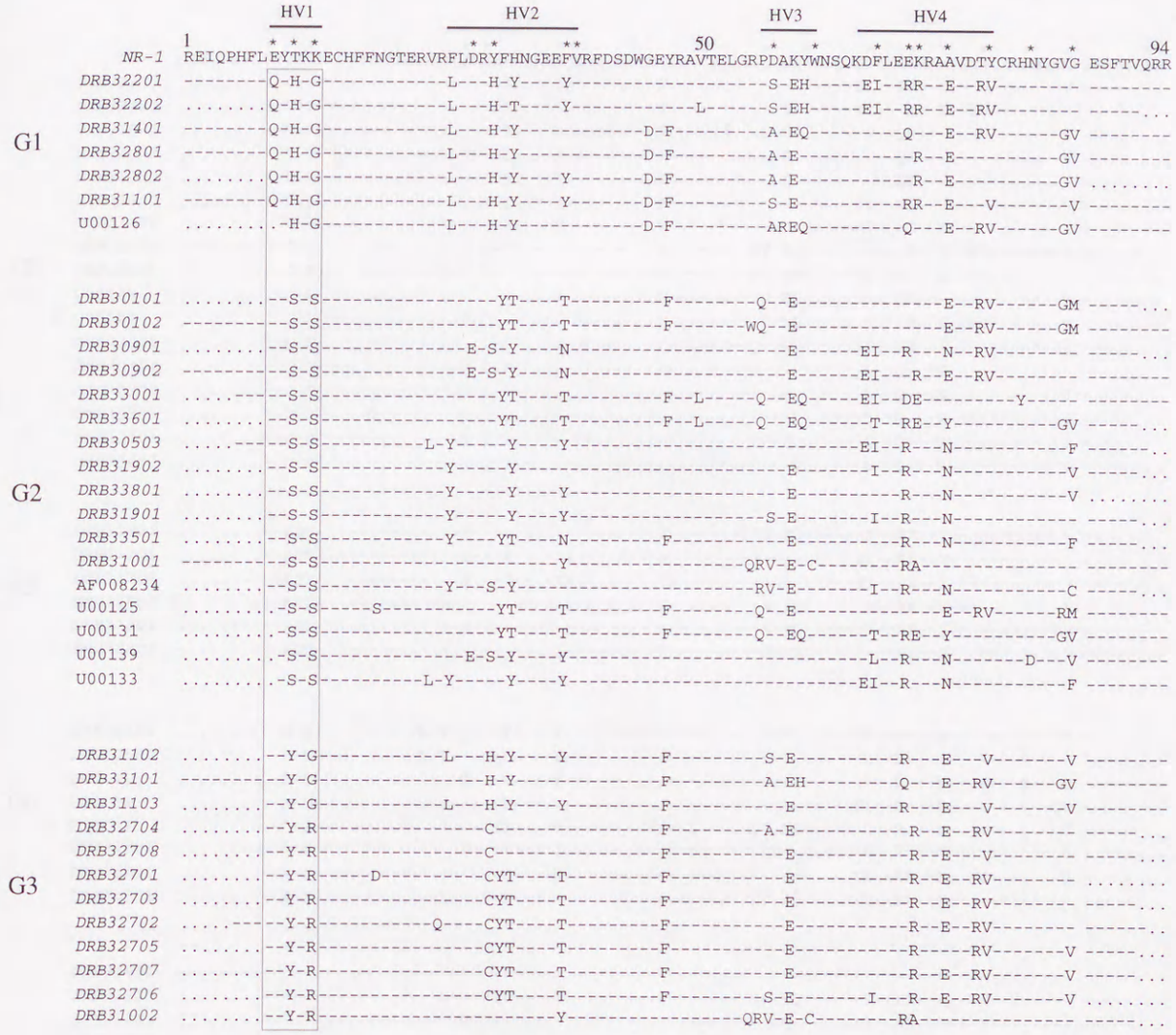


Fig. 1-1 Alignment of the predicted amino acid sequences of the β1 domain encoded by 88 separate *BoLA-DRB3* alleles with amino acids encoded by the NR-1 cDNA clone which represents the *BoLA-DRB3* gene (Aida *et al.*, 1995). Four hypervariable regions, designated HV1 to HV4, are identified. Sequences were divided into eight groups (G1 through G8) according to sequences of amino acids 9-13 in the first hypervariable region HV1. A dash indicates identity with the amino acid sequence at the top. Sequences were taken from the web site of the nternational Society for Animal Genetics (ISAG) BoLA Nomenclature Committee (http://www2.ri.bbsrc.ac.uk/bola/bolafram.htm) and the GenBank nucleotide sequence database (accession numbers U00124, U00135, U00136, U00138, U00142, Z30652 and AF008234) Numbers refer to positions of amino acids in the mature protein. Asterisks above residues indicate residues in the antigen-recognition site in a proposed model of the class II molecule (Brown *et al.* 1993).

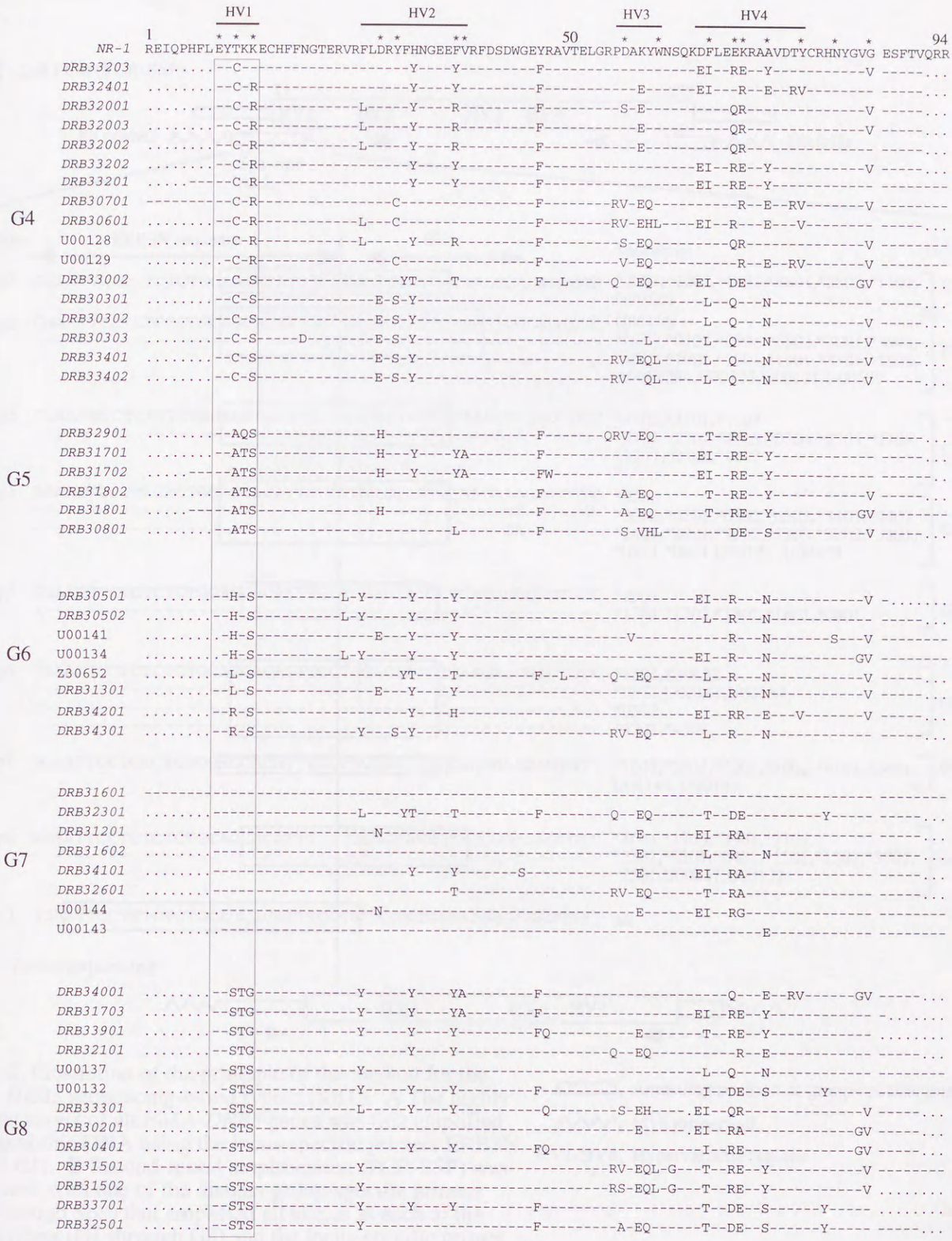


Fig. 1-1 Continued.

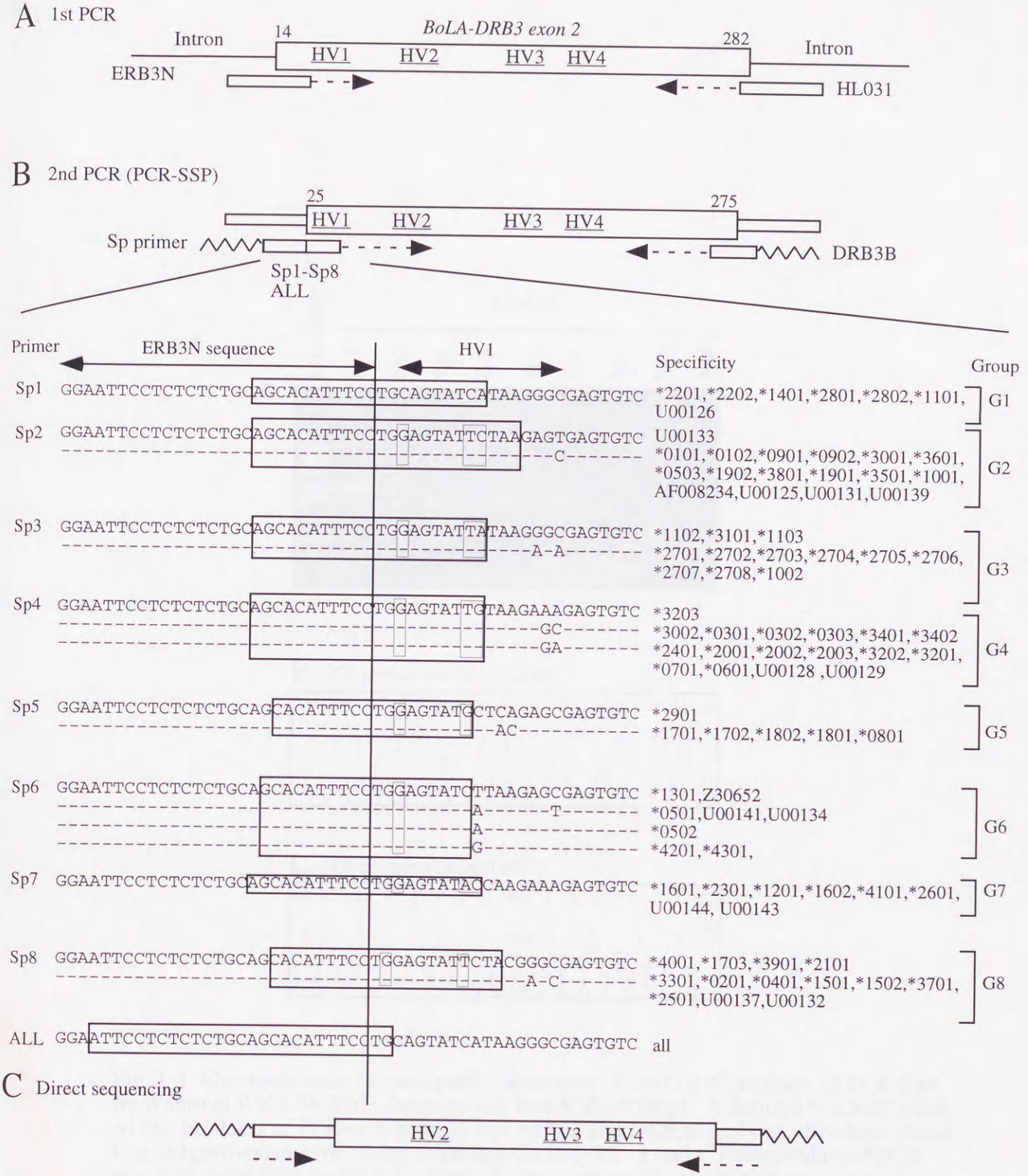


Fig. 1-2 Illustration of the principle of the method for the *BoLA-DRB3* sequencing-based typing (SBT). A The highly variable *exon2* of all *BoLA-DRB3* genes was first amplified from genomic DNA using the locus-specific primers ERB3N and HL031. B Second-round amplification (PCR-SSP) was performed with one of the distinct group-specific primers (Sp1 through Sp8) that amplified all alleles in each of the eight groups (G1 through G8) and the locus-specific primer *DRB3*B. Group-specific primers consisted of nucleotides at the 5' end of ERB3N and had an 8- to 11-bp overlap with *exon2* that encodes the first hypervariable region HV1 and a -21M13 primer. The locus-specific primer DRB3ALL was also used for amplification of all alleles. C The sequencing of each product of the second round of PCR was performed using a -21M13 primer or an M13Reverse primer.

▭, Annealing position of group-specific primer

∧∧∧, M13 primer tail

HV1-HV4, Hypervariable regions

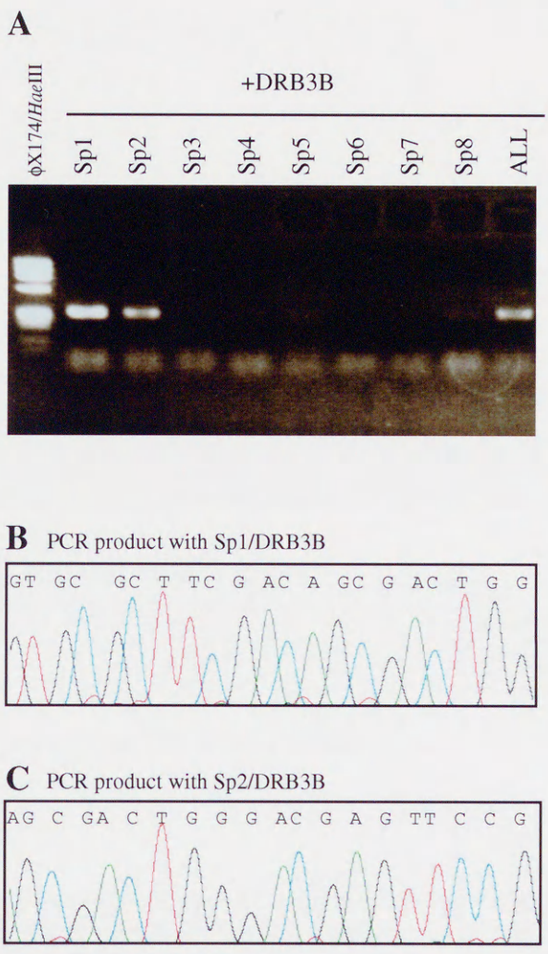


Fig. 1-3 Electrophoresis (A) and partial sequences (B and C) of products of PCR-SSP from animal WK5-38 (Fifth International BoLA Workshop). A Animal WK5-38 yielded two products of PCR with primers Sp1 or Sp2 and *DRB3*B and was, therefore, classified as heterozygous for alleles in groups G1 and G2. B and C Each product of PCR was sequenced with a -21M13 primer. Letters indicate the nucleotide sequence as determined from the chromatograms.

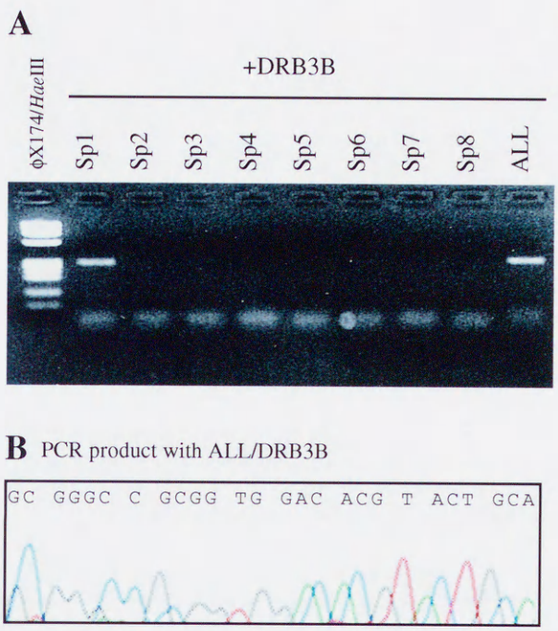


Fig. 1-4 Electrophoresis (A) and partial sequences (B) of PCR-SSP product from animal WK5-42 (Fifth International BoLA Workshop). A Animal WK5-42 yielded one product of PCR with primers Sp1 and *DRB3*B and was, therefore, classified as homozygous or heterozygous for an allele in group G1. B The product of PCR with primers ALL and *DRB3* was sequenced with a -21M13 primer and the animal was classified as homozygous. Letters indicate nucleotide sequence as determined from the chromatograms.

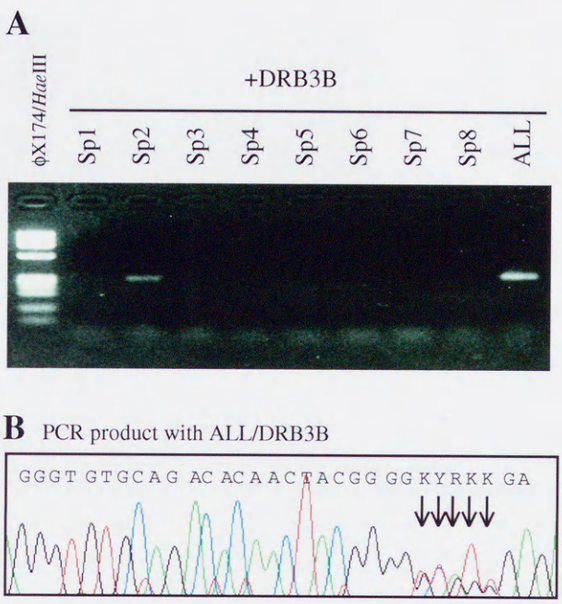


Fig. 1-5 Electrophoresis (A) and partial sequence (B) of the product of PCR-SSP from animal WK5-45 (Fifth International BoLA Workshop). A Animal WK5-45 yielded one product of PCR with primers Sp2 and *DRB3*B and was, therefore, classified as homozygous or heterozygous for an allele in group G2. B The product of PCR with primers ALL and *DRB3* was sequenced with a -21M13 primer and then this animal was classified as heterozygous. Letters indicate the nucleotide sequence as determined from the chromatogram. Arrows indicate theterozygous peaks.

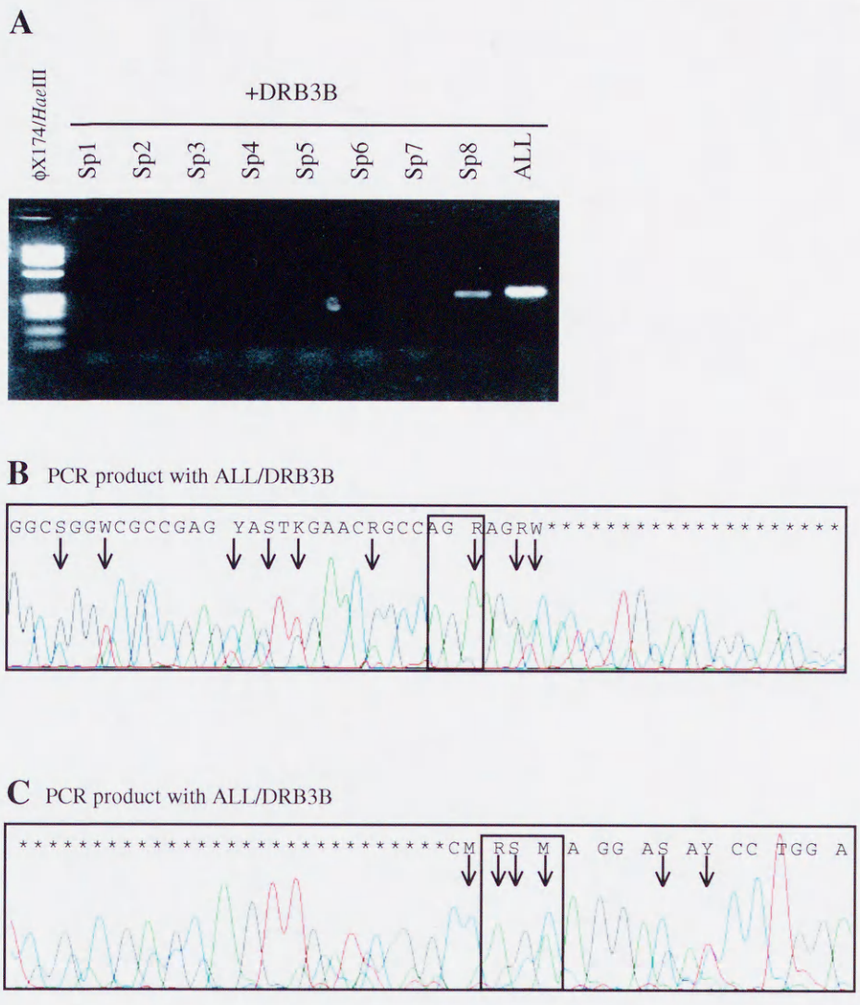


Fig. 1-6 Electrophoresis (A) and direct sequencing (B and C) of the product of PCR-SSP from animal WK5-22 (Fifth International BoLA Workshop). A Animal WK5-22 yielded one product of PCR with primers Sp8 and *DRB3*B and was, therefore, classified as homozygous or heterozygous for an allele in group G8. PCR product with primers ALL and *DRB3* was sequenced using a -21M13 primer (B) and an M13Reverse primer (C). This animal was classified as heterozygous, having an allele with a 3-bp deletion at nucleotide positions 193-195, which corresponds to codon 65 and an allele without this delation. Letters indicate nucleotide sequence as determined from chromatograms. Boxes indicate three peaks at nucleotide positions 193-195. Arrows indicate the heterozygous peaks.

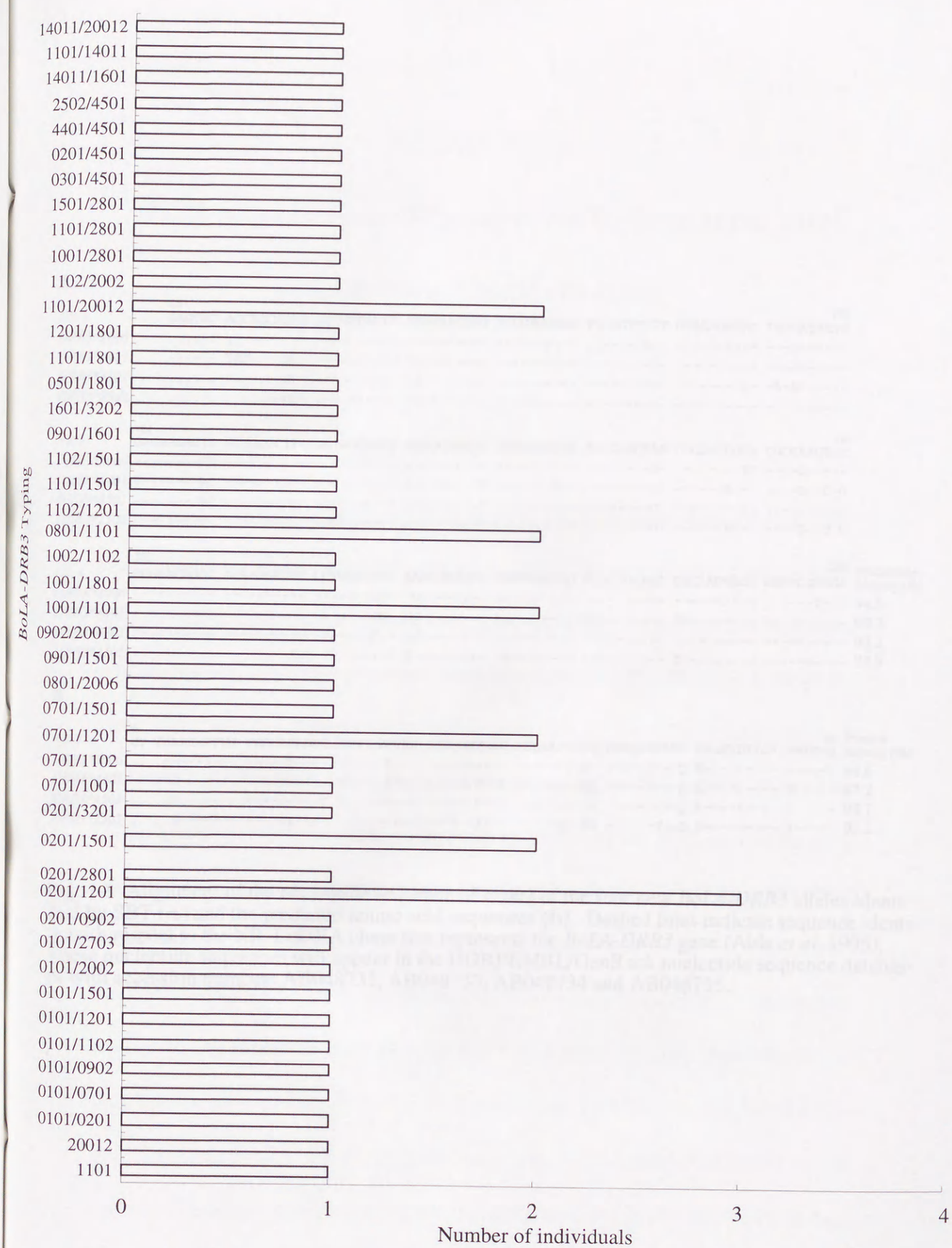


Fig. 1-7 Summary of *BoLA-DRB3* assignments for the 27 alleles that were sequenced by PCR-SBT. The Figure shows all the results of typing and the numbers of samples with each combination of alleles. Twenty-seven different alleles in 43 different heterozygous and two different homozygous combinations were identified in 52 animals.

A

```
                25                                                                                                  100
NR-1            GAGTAT ACCAAGAAAG AGTGTCATTT CTTCAACGGG ACCGAGCGGG TGCGGTTCCT GGACAGATAC TTCCATAATG
DRB3*2006       ...... .--------- ---------- ---------- ---------- -------G-- ---------- ---T------
DRB3*4401       ------ TAT----GC- ---------- ---------- ---------- ---------- ---------- -A-AC-----
DRB3*4501       ...... ...----G-- ---------- ---------- ---------- ---------- --------G- -A-AC-----
DRB3*2502       ...... .....--GC- ---------- ---------- ---------- ------A--- ---------- ----------

                101                                                                                                 180
NR-1            GAGAAGAGTT CGTGCGCTTC GATAGCGACT GGGGCGAGTA CCGGGCGGTG ACCGAGCTAG GGCGGCCGGA CGCCAAGTAC
DRB3*2006       --------CG ---------- --C------- ---------- ---------- --------G- --------TC ----G-----
DRB3*4401       --------AC ---------- --C------- ---------T -------T-- --------G- ------A--- ----G--C-G
DRB3*4501       --------AC ---------- --C------- ---------T ---------- --------G- ---------- ----------
DRB3*2502       ---------- ---------- --C------- ---------T ---------- --------G- ------A--- ----G--C-G

                181                                                                                         260     Nucleotide
NR-1            TGGAACAGCC AGAAGGACTT CCTGGAGGAG AAGCGGGCCG CGGTGGACAC GTACTGCAGA CACAACTACG GGGTCGGTGA        identity (%)
DRB3*2006       ---------- ---------- -------C-- -G-------- ---------- ---------- ---------- ------T---  94.8
DRB3*4401       ---------- ---------- -------CG- GC-------- ---------- ---------- T--------- ----------  90.3
DRB3*4501       ---------- ---------- -------C-- GC-------- ---------- ---------- ---------- ----------  93.2
DRB3*2502       ---------- --------AC ---------C G--------- -T-------- ---------- T--------- ----------  92.9
```

B

```
                9                                                                                   86      Protein
NR-1            EY TKKECHFFNG TERVRFLDRY FHNGEEFVRF DSDWGEYRAV TELGRPDAKY WNSQKDFLEE KRAAVDTYCR HNYGVG    identity (%)
DRB3*2006       .. .--------- -----L---- -Y----R--- ---------- ------S-E- ---------Q R--------- -----V  94.6
DRB3*4401       -- Y-S------- ---------- YT----T--- ------F--L -----Q--EQ ---------R A--------- Y-----  87.2
DRB3*4501       .. .-R------- ---------C YT----T--- ------F--- ---------- ---------Q A--------- ------  94.7
DRB3*2502       .. ..S------- -----Y---- ---------- ------F--- -----Q--EQ ------T--D E--------- Y-----  93.2
```

Fig. 1-8 Alignment of the nucleotide sequence of *exon2* of the four new *BoLA-DRB3* alleles identified by SBT (A) and the predicted amino acid sequences (B). Dashed lines indicate sequence identity with respect to the NR-1 cDNA clone that represents the *BoLA-DRB3* gene (Aida *et al.* 1995). These nucleotide sequences will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB048732, AB048733, AB048734 and AB048735.

CHAPTER 2

PCR-sequence-based typing of *DRB3* gene in the MHC of Japanese Shorthorn cattle

Abstract

A study was made of *exon 2* in the *BoLA-DRB3* of 29 Japanese Shorthorn cattle at the experimental farm, graduate school of agricultural science, Tohoku university (Kawatabi farm), using polymerase chain reaction-sequence-based typing (PCR-SBT). I performed an initial round of PCR using conserved locus-specific primers and a second round of PCR using a locus-specific primer and at least one group-specific primer. I found that the *BoLA-DRB3* alleles of the 29 individuals could be clearly divided into seven independent groups (G1, G2, G4 through G8). Direct sequencing of products of PCR with sequence-specific primers (SSP) revealed that these individuals had nine distinct previously published alleles: *BoLA-DRB3*0101, *0201, *0301, *0501, *0801, *0901, *1101, *1201* and **14011*. The *DRB3*1201* allele was found at the highest frequency (24.1%) and *DRB3*14011* was the second most frequent allele (20.7%). I digested the products of amplification by PCR of *BoLA-DRB3 exon 2* with *Rsa*I, *BstY*I and *Hae*III and identified nine previously described PCR-RFLP patterns (*BoLA-DRB3.2*01, *07, *08, *09, *11, *21, *22, *24* and **27*). The PCR-RFLP patterns reflected the results of PCR-SBT exactly. Our results indicate that *exon 2* of the *BoLA-DRB3* gene is highly polymorphic in Japanese

Shorthorn cattle and that PCR-SBT can improve the resolution and the accuracy of typing of *BoLA-DRB3* alleles in large numbers of animals.

Introduction

The bovine leukocyte antigen (BoLA) system is the major histocompatibility complex (MHC) of cattle. The class II BoLA-DR region is composed of one *DRA* locus and at least three *DRB* loci (Andersson *et al.* 1986a; Muggli-Cocket and Stone 1988, 1989). Moreover, *exon 2* of the *DRB3* gene has been found to be highly polymorphic (Sigurdardottir *et al.* 1988, 1991). The *BoLA-DRB3* gene has been characterized by serology (Davies and Antczak 1991b), mixed lymphocyte culture (Davies and Antczak 1991a), isoelectric focusing (IEF) (Watkins *et al.* 1989; Glass *et al.* 1992), restriction fragment length polymorphism (RFLP) analysis (Andersson *et al.* 1986b), PCR-RFLP (van Ejik *et al.* 1992), T cell typing with cell lines and clones (Rothel *et al.* 1990), heteroduplex analysis (Sitte *et al.* 1995), sequence-specific oligonucleotide (SSO) typing (Sitte *et al.* 1996), denaturing gradient gel electrophoresis (DGGE) (Aldrige *et al.*, 1998), analysis of a microsatellite adjacent to *DRB3* (Ammer *et al.* 1992; Ellegren *et al.* 1993), and sequencing of genomic DNA, cDNA or cloned products of PCR (Aida *et al.* 1995; Burke *et al.* 1991; Muggli-Cockett and Stone 1989; Groenen *et al.* 1990; Sigurdardottir 1991; Xu *et al.* 1993b). However, most of these methods are limited in their ability to define new alleles, a severe drawback in examinations of polymorphic loci. Therefore, we recently developed a more precise method for PCR-sequence-based typing (SBT) which allows identification of specific *BoLA-DRB3* alleles in large numbers of animals (Takeshima *et al.*, in press).

To date more than 102 *BoLA-DRB3* alleles have been identified by DNA sequencing of *exon 2* from various breeds of cattle (Groenen *et al.* 1990; Davies *et al.* 1994a; Russell *et al.* 1997; Takeshima *et al.*, in press; http://www2.ri.bbsrc.ac.uk/bola/). However, no information is available, to our knowledge, of these alleles in Japanese Shorthorn cattle, which arose from crosses between Shorthorn cattle and Nanbu cattle and are raised in the northern region in Japan. The present study was designed to determine the nucleotide sequences of *exon 2* of *BoLA-DRB3* alleles in a herd of Japanese Shorthorn cattle using PCR-SBT and to compare the results with *BoLA-DRB3* haplotypes, as determined by PCR-RFLP. The results confirmed the usefulness of the PCR-SBT strategy that we had developed previously for analysis of *BoLA-DRB3 exon 2*.

Materials and Methods

Animals and preparation of DNA

Twenty-nine Japanese Shorthorn cattle from the Kawatabi Farm of the University of Tohoku in Miyagi, Japan, were analyzed (Table 2-1). Genomic DNA was prepared from whole blood as described previously (Takeshima *et al.*, in press).

Typing by PCR with sequence-specific primers (PCR-SSP) and nucleotide sequencing

The first round of amplification by PCR was performed with the locus-specific primers ERB3N (Takeshima *et al.*, in press) and HL031 (van Eijk *et al.* 1992) and subsequent amplification by PCR-SSP was performed with distinct, group-specific 3' primers (Sp1 through Sp8) (Takeshima *et al.*, in press) and a locus-specific 5' primer DRB3B (Takeshima *et al.*, in press). In addition, the

locus-specific 3' primer, DRB3ALL, which allowed amplification of the alleles in all previously identified groups was used (Takeshima *et al.,* in press).

Reactions performed with 20 ng of DNA (1 μl) in a final volume of 50 μl. The reaction mixture contained 49 μl of PCR buffer [final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 0.1% TritonX-100], with 120 μM dNTPs, 1.5 mM $MgCl_2$, 200 μM each primer and 1 unit of recombinant rTaq DNA polymerase (Toyobo co., Ltd., Osaka, JP). The thermal cycling profile for the first round of amplification included an initial denaturation for 5 min at 95℃, which was followed by 20 cycles of 50 sec at 95℃, 50 sec at 60℃ and 50 sec at 72℃. The final extension reaction was allowed to proceed for 10 min at 72℃. Subsequently, 1-μl aliquots of the mixture after the first PCR were transferred to cells of a 96-well plate. Each well contained 24 μl of GeneAmp^R Gold Buffer [final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% TritonX-100] plus 120 μM dNTPs, 1.5 mM $MgCl_2$, 400 μM each primer and 1 unit of Taq polymerase (AmpliTaq^{TM} Gold, Applied Biosystems, Foster City, CA) for a PCR-SSP with the following thermal cycling profile: initial denaturation for 10 min at 95℃, followed by 20 cycles of 1 min at 95℃, 30 sec at 64℃ and 30 sec at 72℃, with final extension for 5 min at 72℃. All reactions were performed in a TGRADIENT Thermocycler 96 (Biometra, Goettingen, DE).

After PCR-SSP, 5 μl of the reaction mixture were fractionated by electrophoresis on a 2% agarose/TAE gel. The gel was stained with ethidium bromide and bands of DNA were visualized under UV light.

A CEQ^{TM}2000 Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., Fullerton, CA) was used to sequence each product of PCR-SSP with a -21-mer M13 universal primer, 5'-TGT AAA ACG ACG GCC AGT-3' or an M13 reverse primer, 5'-CAG GAA ACA GCT ATG

ACC-3'. I combined 6 μl of the reaction mixture after the second-round PCR, which had been diluted 10-fold by addition of distilled water, 1 μl of a solution of 12 pmol/μl sequencing primer, 2 μl of 10x Sequencing Reaction Buffer, 1 μl of dNTP Mix, 2 μl of ddUTP Dye Terminator, 2 μl of ddGTP Dye terminator, 2 μl of ddCTP Dye Terminator, 2 μl of ddATP Dye Terminator, and 1 μl of the solution of DNA polymerase. Then cycle sequencing reactions were performed as described in the manual from the manufacturer of the kit. Reaction mixtures were applied to the CEQ™ 2000 DNA analysis system (Beckman Coulter Inc.). Data were collected with the software package provided with the CEQ™2000 sequencing system. In addition, sequencing of part of each product of PCR was performed with a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) as described previously (Takeshima *et al.*, in press).

Analysis of data

Raw data after chromatography were collected with Sequencing Analysis™ and Factura™ programs (Applied Biosystems). Nucleotide sequences were compared with sequences in the GenEMBL database using the BLAST (Gish and States 1993) algorithm.

PCR-RFLP

The *DRB3 exon 2* fragment was amplified firstly by the PCR using the locus-specific primers ERB3 (Nagaoka *et al.* 1999) and HL031 (van Eijk *et al.* 1992) and then it was amplified with the locus-specific primers ERB3 and SRB3 (Nagaoka *et al.* 1999). Each product of PCR was incubated for 4 h at 37℃ with three units of *Rsa*I or *Hae*III in a final volume of 20 μl, or for 1

h at 60℃ with three units of *Bst*YI in a total of 20 μl. Restriction fragment patterns were examined after electrophoresis on a 12% polyacrylamide gel or a 5% agarose/TAE gel.

Results and Discussion

PCR-SSP typing and subsequent direct sequencing of BoLA-DRB3 alleles

The genotypes of 29 Japanese Shorthorn cattle were determined for *exon 2* of the *BoLA-DRB3* allele by PCR-SSP typing. As shown in Fig. 2-1, *exon 2* of the *BoLA-DRB3* gene was successfully amplified by two-step PCR in all cases and the *BoLA-DRB3* alleles that were identified could be clearly devided into seven independent groups (G1, G2, G4 through G8) after amplification in eight second PCRs (Tables 2-1 and 2-2). In 24 cases, *exon 2* of the *BoLA-DRB3* gene was amplified as two separate products of PCR, with 12 different combinations of groups. These individuals were judged to be heterozygous. By contrast, five animals, numbers 802, 824, 846, 847 and 865, each generated only one product of PCR. Moreover, second-round PCR for all individuals generated a visible product when the locus-specific primer pair DRB3ALL and DRB3B was used (Fig. 2-1). All samples of DNA were analyzed twice and the reproducibility of the results of PCR-SSP was 100% for all animals.

For the 24 animals that had been judged to be heterozygous by PCR-SSP typing, we determined the sequence of each product of PCR using the -21-mer M13 universal primer and the CEQ™2000 system (Table 2-1). By contrast, for the five animals that each generated a simple product of PCR, to avoid missing the amplification of a new allele and any risk of loss of one of the haplotypic alleles, we analyzed the product of PCR that had been amplified with the ALL and

DRB3B primers by direct sequencing (Table 2-1). Sequencing in these cases was performed with the Big-Dye Terminator Cycle Sequencing system, which has considerably higher sensitivity than the CEQ™2000 system used for the previous 24 animals. Only these animals, namely, 802, 824 and 846, gave a homozygous pattern, while animals, 847 and 865, gave typical heterozygous patterns.

Using PCR-SBT, we identified nine different *BoLA-DRB3* alleles (Table 2-2) in 15 different heterozygous and two different homozygous combinations (Table 2-1). To date, 102 alleles have been identified by sequencing of cloned genomic DNA, cDNA or cloned products of PCR (Groenen *et al.* 1990; Davies *et al.* 1994a; Russell *et al.* 1997; http://www2.ri.bbsrc.ac.uk/bola/) and by PCR-SBT of genomic DNA (Takeshima *et al.*, in press) from various breeds of cattle that include Holstein, Red Pied, Martinique, Brahman, Japanese Black, Jersey, Hereford, Ethiopian Arsi, N'Dama, Boran, Swedish Red-and-White and Simmental. Therefore, we compared the nine alleles found in Japanese Shorthorn cattle with the previously identified *BoLA-DRB3* alleles. The sequences of all of the nine alleles appeared to be identical to those of previously identified *BoLA-DRB3* alleles (Table 2-4). No new sequences were identified. In addition, the concordance between the results of analysis by PCR-SBT and PCR-SSP was 100% for all in 29 animals.

Although ours was not a random sample of cattle, we were still interested in the frequencies of the nine *BoLA-DRB3* alleles (Table 2-3). *BoLA-DRB3*1201* was the most frequent allele in our sample (24.1%), followed by **14011* (20.7%). The two alleles also occurred at a high frequency in cattle born from cows derived from populations in Iwate prefecture. Nine of twelve animals that were heterozygous for *BoLA-DRB3*14011* were offspring of "Kazutono" and a certain cow, as shown in Table 2-1. By contrast, two alleles (*BoLA-DRB3*0201* and **0901*) were

present a frequencies of less than <2%. A significant difference in the frequencies of *BoLA-DRB3* alleles was also apparent between progeny of cows from Aomori prefecture and Iwate prefecture. For example, the *BoLA-DRB3*0101* accounted for 8.3% and 20.6%, respectively, of the alleles in cattle whose mothers originated in Aomori or Iwate. The most frequently detected allele in twelve offspring of mothers from Aomori was *BoLA-DRB3*0801* and this allele accounted for about 2.9% of the alleles in the progeny of dams from Iwate.

PCR-RFLP of BoLA-DRB3 alleles

Recently, van Eijk *et al.* (1992), Gelhaus *et al.* (1995) and Maillard *et al.* (1999) examined and distinguished between 54 *BoLA-DRB3.2* alleles by PCR-RFLP analysis using the restriction enzymes *Rsa*I, *Hae*III and *Bst*YI. An additional new allele was been defined as pattern 11 in the DNA from Jersey cattle (Gilliespie *et al.* 1999). In the present study, to type *BoLA-DRB3.2* alleles by PCR-RFLP, we amplified genomic DNA by PCR using the locus-specific primers ERB3 and HL031 and then the locus-specific primers ERB3 and SRB3. The 292-base pair fragment PCR amplified from case of the *BoLA-DRB3* gene was then digested with the restriction enzymes mentioned above. Analysis of the *BoLA-DRB3.2* fingerprints of our 29 Japanese Shorthorn cattle resulted in identification of nine of the 65 previously identified alleles with frequencies ranging from 24.1% to 1.7% (Tables 2-1 and 2-4). As in the case of PCR-SBT typing, no new PCR-RFLP was detected. All of the PCR-RFLP patterns identified exactly matched the patterns predicted from the DNA sequences determined by PCR-SBT.

Collectively, the results obtained by PCR-SBT and PCR-RFLP typing in our 29 Japanese Shorthorn cattle indicated that our method of PCR-SBT is useful and has potential clinical application in cases when high-volume typing of the *BoLA-DRB3.* gene is necessary.

Relationships between results of PCR-SSP, PCR-SBT and PCR-RFLP analysis in defining BoLA-DRB3 alleles

As shown in Table 2-2, nine sequenced *BoLA-DRB3* alleles found in 29 Japanese Shorthorn cattle were identified as nine allelic types by PCR-RFLP, but only seven of these types were distinguished by PCR-SSP. However, typing of *BoLA-DRB3.2* alleles by PCR-RFLP has been suggested to be less than fully informative because 12 of 42 restriction fragment patterns from 52 animals characterized at the Fifth International BoLA Workshops corresponded to more than a single allelic sequence (Gelhaus *et al.* 1995). In deed, recent our work showed that only 20 of 27 sequenced *BoLA-DRB3* alleles had been distinguished by PCR-RFLP at the Fifth International BoLA Workshop (Takeshima *et al.,* in press). Therefore, for comparison, we gathered all the available data on *BoLA-DRB3* alleles in terms of deduced restriction fragment patterns or deduced PCR-SSP types for the 102 alleles that had been defined solely in terms of DNA sequence (Table 2-4).

Only 50 of the 102 sequenced *BoLA-DRB3* alleles were distinguished by PCR-RFLP, and, in addition, only 28 alleles (*BoLA-DRB3.2*5, *7, *8, *17, *19, *21, *26, *28, *29, *31, *32, *33, *35, *36, *37, *38, *42, *43, *44, *46, *47, *48, *50, *51, *52, *54, *fah* and **yab*) out of these PCR-RFLP types corresponded to a single sequence. Conversely, with the combination of PCR-RFLP

and PCR-SSP together, 17 *BoLA-DRB3* alleles could additionally be identified as independent alleles without sequencing, namely, *BoLA-DRB3*1001, *3* and G2; *BoLA-DRB3*1002, *3* and G3; *BoLA-DRB3*1302, *9* and G6; *BoLA-DRB3*46011, *13* and G4; *BoLA-DRB3*0401, *13* and G8; *BoLA-DRB3*4501, *15* and G3; *BoLA-DRB3*4401, *20* and G3; *BoLA-DRB3*2901, *20* and G5; Z30652, **20* and G6; *BoLA-DRB3*2301, *20* and G7; *BoLA-DRB3*1101, 22* and G1; *BoLA-DRB3*3101, *27* and G3; *BoLA-DRB3*3001, *34* and G2; *BoLA-DRB3*3002, *34* and G4; *BoLA-DRB3*0502, *41* and G6; *BoLA-DRB3*1902, *53* and G2; and U00137, **53* and G8. The remaining 18 PCR-RFLP types corresponded to many allelic sequences. Thus, since PCR-RFLP typing remains less than fully informative because several allelic nucleotide substitutions cannot be identified with the restriction enzymes available to date, it appears that PCR-SBT is the most sensitive method for identification of alleles.

Our present study demonstrated that the *BoLA-DRB3* locus is highly polymorphic in Japanese Shorthorn cattle. A certain degree of *BoLA-DRB3* polymorphism has been reported in studies of Holstein, Jersey and Argentine Creole cattle (Gilliespie *et al.* 1999; Dietz *et al.* 1997a; Dietz *et al.* 1997b; Giovambattista *et al.* 1996). However, there are significant differences in allelic frequencies of *BoLA-DRB3.2* alleles between Holstein, Jersey, Argentine Creole and Japanese Shorthorn cattle. For example, the six most frequently detected alleles in Jersey cows were *BoLA-DRB3.2*8, *10, *15, *21, *36* and ibe, accounting for approximately 74% of the alleles in the population of the herd that was examined (172 animals). Moreover, the six most frequently detected alleles (*BoLA-DRB3.2*8, *11, *16, *22, *23* and **24*) accounted for 70.3% of the alleles in a population of 127 Holstein cows (Dietz *et al.* 1997b). The six most frequently detected alleles in Argentine Creole cows (194 animals) were *BoLA-DRB3.2*15, *18, *24, *20,*

27 and *5* and these accounted for approximately 73% of the alleles in the herd. By contrast, approximately 93% of the alleles in our sample of Japanese Shorthorn cattle were accounted for by six alleles (*BoLA-DRB3.2*8*, **27*, **24*, **9*, **21* and **1*) and these alleles correspond to *BoLA-DRB3*1201*, **14011*, **0101*, **0301*, **0801* and **0501*, respectively. Only two of six alleles that occurred at high frequency in Holstein, Jersey and Argentine Creole breeds were found in our Japanese Shorthorn cattle. Collectively, results of the present and previous studies indicate that differences exist between breeds of cattle with regard to frequencies of specific *BoLA-DRB3* alleles.

Further sequencing is needed so that the extent of *BoLA-DRB3* polymorphism can be more accurately defined. I recently developed a new method for PCR-SBT of *BoLA-DRB3* alleles that allows detection of as yet undefined alleles that are composed of new combinations of known sequences (Takeshima *et al.*, in press). In the present study, we showed that PCR-SBT is reliable and rapid, and does not require cloning of alleles prior to nucleotide sequencing. Therefore, it offers distinct advantages over previous techniques for *BoLA-DRB3* typing.

Table 2-1

Results of *BoLA-DRB3* typing by PCR-SBT, PCR-SSP and PCR-RFLP in analysis of 29 Japanese Shorthorn cattle

Animal number	Sire	Dam (origin)	PCR-SBT*		PCR-SSP*		PCR-RFLP*	
			A	B	A	B	A	B
196	676	65 (Aomori)	*0501	*0801	G6	G5	*01	*21
297	210	196 (〃)	*0101	*0501	G2	G6	*24	*01
326	210	192 (〃)	*0301	*1201	G4	G7	*09	*08
347	Akihebi	202 (〃)	*0101	*1201	G2	G7	*24	*08
356	Sachitama	289 (〃)	*0301	*0801	G4	G5	*09	*21
371	Ohno268	Kiyofuku (〃)	*0801	*0901	G5	G2	*21	*11
373	Rikihoho	Tadahime (〃)	*0201	*0801	G8	G5	*07	*21
374	Sachikaze	Okahime91 (〃)	*0801	*1201	G5	G7	*21	*08
375	Matsunaka	Fujihime (〃)	*1201	*14011	G7	G1	*08	*27
376	Kazutono	356 (〃)	*0801	*14011	G5	G1	*21	*27
384	Kazutono	326 (〃)	*0301	*1201	G4	G7	*09	*08
385	Sachitama	250 (〃)	*0301	*14011	G4	G1	*09	*27
802	Akihebi	712 (Iwate)	*1201	*1201	G7	G7	*08	*08
807	210	616 (〃)	*0101	*0301	G2	G4	*24	*09
813	210	780 (〃)	*0101	*1201	G2	G7	*24	*08
814	210	693 (〃)	*0301	*0501	G4	G6	*09	*01
824	210	710 (〃)	*0101	*0101	G2	G2	*24	*24
829	210	788 (〃)	*0101	*0301	G2	G4	*24	*09
846	Kotohoho	802 (〃)	*1201	*1201	G7	G7	*08	*08
847	Sachitama	788 (〃)	*1101	*14011	G1	G1	*22	*27
849	Sachitama	787 (〃)	*0501	*0801	G6	G5	*01	*21
863	Kazutono	809 (〃)	*0301	*14011	G4	G1	*09	*27
864	Kazutono	829 (〃)	*0101	*14011	G2	G1	*24	*27
865	Kazutono	788 (〃)	*1101	*14011	G1	G1	*22	*27
866	Kazutono	846 (〃)	*1201	*14011	G7	G1	*08	*27
875	Kazutono	813 (〃)	*1201	*14011	G7	G1	*08	*27
877	Kazutono	802 (〃)	*1201	*14011	G7	G1	*08	*27
878	Kazutono	802 (〃)	*1201	*14011	G7	G1	*08	*27
879	Kazutono	788 (〃)	*0101	*14011	G2	G1	*24	*27

*Both alleles of each animal, A and B, are shown.

Table 2-2
Comparative *BoLA-DRB3* typing of 29 Japanese Shorthorn cattle by PCR-SBT, PCR-SSP and PCR-RFLP analysis

PCR-SSP	PCR-SBT	PCR-RFLP
G1	*14011	*27
	*1101	*22
G2	*0101	*24
	*0901	*11
G4	*0301	*09
G5	*0801	*21
G6	*0501	*01
G7	*1201	*08
G8	*0201	*07

Table 2-3
Frequencies of various *BoLA-DRB3* alleles in 29 Japanese Shorthorn cattle as determined by PCR-SBT

DRB3 allele	Frequency*					
	Total (n=58)		Aomori (n=24)		Iwate (n=34)	
	n	(%)	n	(%)	n	(%)
*1201	14	(24.1)	5	(20.8)	9	(26.5)
*14011	12	(20.7)	3	(12.5)	9	(26.5)
*0101	9	(15.5)	2	(8.3)	7	(20.6)
*0301	8	(13.8)	4	(16.7)	4	(11.8)
*0801	7	(12.1)	6	(25.0)	1	(2.9)
*0501	4	(6.9)	2	(8.3)	2	(5.9)
*1101	2	(3.4)	0	(0.0)	2	(5.9)
*0201	1	(1.7)	1	(4.2)	0	(0.0)
*0901	1	(1.7)	1	(4.2)	0	(0.0)

*The animals used in this study were born to cows that originated from Aomori Prefecture or Iwate Prefecture.

Table 2- 4

BoLA-DRB3 alleles with their equivalents*

PCR-RFLP type	Fragment patterns (*Rsa*I, *Bst*YI, *Hae*III)			PCR-SSP type	*DRB3* allele by PCR-SBT
*01†	a	a	a	G2	*0503, U00133
				G6	*0501†, U00134
*02	b	b	a	G6	U00141, *1301
*03	b	b	b	G2	*1001
				G3	*1002
*04	c	a	a	–	No sequence
*05	r	c	c	G8	*3301
*06	d	a	a	G1	*2201, *2202
*07†	e	c	c	G8	*0201†
*08†	f	a	a	G7	*1201†
*09†	f	d	a	G4	*0301†, *0302
				G6	*1302
*10†	f	b	a	G7	*1601, *1602, U00143, *2006
*11	g	e	a	G2	*0901†, *0902
*12	h	a	a	G4	*3201, *3202, *3203
				G5	*1701, *1702
*13	h	b	a	G4	*46011
				G8	*0401
*14	h	b	b	–	No sequence
*15	i	b	a	G3	*4501
				G4	*20011, *20012, *2002, *2003, *2004
				G8	AF144546
*16	j	b	d	G8	*1501, *1502
*17	k	b	b	G2	*2502
*18	l	b	f	G5	*1801, *1802
*19	s	b	b	G7	*2601
*20	l	b	b	G2	*3601, U00131
				G3	*4401
				G5	*2901
				G6	Z30652
				G7	*2301
*21†	l	b	e	G5	*0801†
*22†	m	b	a	G1	*1101†
				G3	*1102, *1103, *4002
*23	n	b	a	G3	*2701, *2702, *2703, *2705, *2706, *2707, *2708, *2709, *2710
*24†	n	b	b	G2	*0101†, *0102, U00125
*25	o	a	a	–	No sequence
*26	o	a	b	G4	*0601
*27†	o	b	f	G1	*14011†, *14012, U00126
				G3	*3101
*28	o	b	b	G4	*0701
*29	p	c	c	G7	*4101
*31	i	b	f	G1	*2801
*32	m	a	a	G4	*2401
*33	n	b	f	G3	*2704
*34	l	a	b	G2	*3001
				G4	*3002
*35	c	b	b	G8	*2101
*36	l	b	a	G4	U00128
*37	o	b	a	G4	U00129
*38	b	d	a	G2	U00139
*39	t	b	a	–	No sequence
*40	u	b	a	–	No sequence
*41	a	b	a	G2	*3801, *1901
				G6	*0502
*42	h	b	f	G1	*2802
*43	k	b	f	G8	*25012
*44	k	b	i	G8	*25011
*45	s	d	b	G4	*3401, *3402
*46	v	b	a	G2	*3501
*47	w	a	a	G8	*1703
*48	w	b	a	G8	*3901
*49	w	b	e	G8	*3701, U00132
*50	x	b	a	G8	*4001
*51	g	a	a	G6	*4201
*52	s	d	a	G4	*0303
*53	y	b	a	G2	*1902
				G8	U00137
*54	j	d	b	G6	*4301
fah	f	a	h	G7	U00144
ydb	y	d	b	G2	AF008234
fab	f	a	b	–	No sequence
fbb	f	b	b	–	No sequence
fbe	f	b	e	–	No sequence
iaa	i	a	a	–	No sequence
iae	i	a	e	–	No sequence
kba	k	b	a	–	No sequence
nbe	n	b	e	–	No sequence
obe	o	b	e	–	No sequence

*To date, 102 alleles have been identified by sequencing of cloned genomic DNA, cDNA or cloned products of PCR and by PCR-SBT of genomic DNA. These alleles were identified in various breeds that included Holstein, Red Pied, Martinique, Brahman, Japanese Black, Jersey, Hereford, Ethiopian Arsi, N'Dama, Boran, Swedish Red-and-White, Simmental.

† *BoLA-DRB3* alleles and PCR-RFLP types found in 29 Japanese Shorthorn cattle in this study.

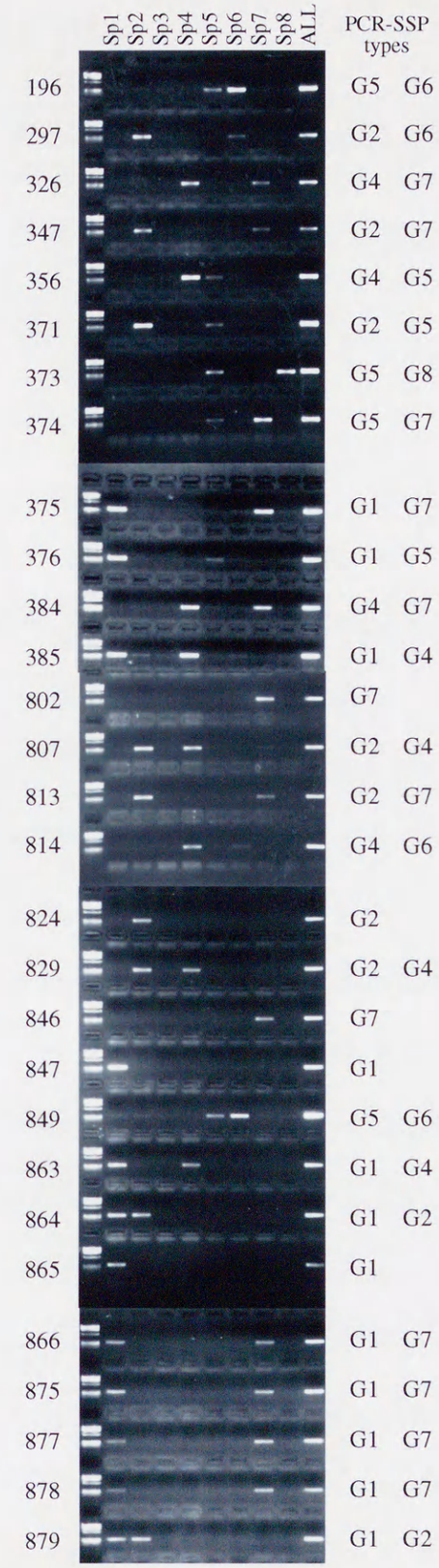


Fig. 2-1 Electrophoresis of products of PCR-SSP specific for *exon2* of *BoLA-DRB3*ALLele in a herd of Japanese Shorthorn cattle. The highly variable *exon2* of all *BoLA-DRB3* genes was first amplified from the genomic DNA by PCR with the locus-specific primers ERB3N and HL031. Second-round amplification (PCR-SSP) was performed with one of the distinct group-specific primers (Sp1 through Sp8) that amplified all the alleles in each of the eight groups (G1 through G8) and the locus-specific primer *DRB3*B. The locus-specific primer DRB3ALL (ALL) was also used for amplification of all alleles. The numbers on the left correspond to the numbers in Table 2-1.

CHAPTER 3

Variation and evolution of bovine MHC class II *DRB3* genes in Japanese Black, Japanese Shorthorn, Jersey and Holstein cattle

Abstract

The allele frequencies of MHC gene in different natural cattle populations allows to differentiate and reconstruct genetic distance between populations, thereby providing the molecular basis for specification of possible common origin of populations. The genotypes of 419 samples from four different Japanese cattle populations were determined for *exon 2* of the *BoLA-DRB3* allele by polymerase chain reaction-sequence-based typing (PCR-SBT). The 35 different previously published alleles and the three novel alleles were identified: 20 published alleles from 102 Japanese Shorthorn; 12 published alleles from 17 Jersey; 18 published alleles from 100 Holstein; and 24 alleles including 3 new alleles from 200 Japanese Black. To differentiate the allelic variations between four distinct breeds, the gene frequencies of the *BoLA-DRB3* alleles in each breed were compared with those of other populations. All breeds examined were found to carry extremely high *DRB3* diversities, with heterozygosity rates of between 90.5% and 100%, and, in addition, the remarkably dissimilar distribution patterns of *BoLA-DRB3* alleles between other breeds. Moreover, it appeared that the *BoLA-DRB3* sequences isolated from single breed were

shared between other breeds. This result confirmed by a phylogenetic tree constructed from *DRB3* nucleotide sequences. Moreover, the phylogenetic tree constructed based on the allele frequencies of the *BoLA-DRB3* in these breeds using the Neighbour-joining method suggested that Holstein and Japanese Black were the closest to each other, but Jersey was farther from the these both breeds than Japanese Shorthorn.

Introduction

The major histocompatibility complex (MHC) class I and class II molecules exhibit an extraordinary high degree of genetic polymorphism in many vertebrate species (Klein. 1986). The MHC polymorphism occurs predominantly at residues involved in peptide binding (Bjorkman *et al.* 1987; Brown *et al.* 1988), and there is compelling evidence that the polymorphism is maintained by some form of balancing selection (Hughes and Nei. 1988, 1989). The essential role of the MHC molecules for immunological recognition of foreign peptide antigens implies that the cause for this selection is related to the influence of MHC polymorphism on host defense against pathogens (Bodmer 1972; Doherty and Zinkernagel 1975).

MHC variability in natural population is of great interest to evolutionary biologists because of the high levels of polymorphism typically observed. Consequently, representative species of several mammalian orders including Artiodactyla, Carnivora, Cetacea, Primates, and Rodentia have been characterized for MHC allelic diversity. Some species exhibit extremely high levels of MHC polymorphism (Mikko and Andersson, 1995a; Slierendregt *et al.* 1995; Swaebrigk *et al.* 1995; Van Den Bussche *et al.* 1999; Yuhki and O' Brien 1997), whereas others are characterized by

very low levels (Elegren *et al.* 1993; Mikko and Andersson 1995b; Murray *et al.* 1998). Even in species characterized by high levels of MHC polymorphism, some populations are monomorphic or oligomorphic (Klein 1987). The trans-species polymorphism hypotheses seems to provide as universal explanation for difference in levels of MHC variability and other characters of the MHC in natural populations (Klein *et al.* 1993). According to this hypothesis, variability within and among populations of species is directly related to the interplay between effective populations size, time of divergence, and intensity of selection acting at a particular locus (Klein 1987). Thus, the allele frequencies of MHC gene in different population allows to differentiate and reconstruct genetic distance between populations, thereby providing the molecular basis for specification of possible common origin of populations.

Around the second century A. D., cattle migrated from North China via the Korea peninsula to Japan. This cattle movement was accompanied by the introduction of rice cultivation. Both genetic (Namikawa, 1980) and morphological (Ogawa *et al.* 1989) studies have illustrated that native Japanese cattle are *B. taurus* and are representatives of the " Turano-Mongolia" type (Felius 1995). Afterward, at the end of the 19th century, cattle of several European breeds were imported into Japan for the purpose of upgrading native breeds. The Jersey cattle breed originated from a small island and have existed in effective genetic isolation because of strict breeding practices or ~200 years (French *et al.* 1996) and the Holstein cattle breed which is one of dairy cattle originated from Netherlands. It is possible that some of these exotic genetic materials retain an influence in modern Japanese cattle. Japanese Black cattle, which is the majority of beef breeds in Japan, is less influenced by European breeds than other breeds in Japan and other countries. Japanese

Shorthorn cattle arose from crosses between Shorthorn cattle and Nanbu cattle and are raised in the northern region in Japan.

In cattle there is one predominantly expressed class II *DRB* locus, denoted *DRB3* (Lewin 1996), which is also the most polymorphic class II locus in cattle. Although it has now reported that 65 *BoLA-DRB3.2* alleles in *exon 2* were distinguished by the polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) method described by van Eijk *et al.* (1992), this PCR-RFLP typing has been found to be limited in detection alleles which are further split into several multiple alleles at the nucleotide level. In deed, 89 alleles have been identified by sequencing of cloned genomic DNA, cDNA or cloned products of PCR (Aida *et al.* 1995; Davies *et al.* 1997; Maillard *et al.* 1999; Russell *et al.* 1997, 2000) from various breeds of cattle. Recently, we developed a more precise new method for PCR-sequence-based typing (SBT) which allows identifications of specific *BoLA-DRB3* alleles in large numbers of animals and identified additional 4 new alleles and the 27 previously published alleles (Takeshima *et al.* in press). The present study was designed to determine the nucleotide sequences of *exon 2* of *BoLA-DRB3* alleles in a total of 419 animals from four distinct cattle breeds such as Japanese Black, Japanese Shorthorn, Holstein and Jersey by our PCR-SBT method, and the allele frequencies and the phylogenetic relationship of *BoLA-DRB3* alleles and the genetic background of these populations are discussed.

Materials and Methods

Animals and extraction of DNA

A total of 419 samples kindly offered by Livestock Improvement Association (Tochigi, Japan) were examined for distribution of *BoLA-DRB3* alleles: 200 heads of Japanese Black cattle; 100 heads of Japanese Shorthorn cattle; 102 heads of Holstein cattle; and 17 heads of Jersey cattle. High-molecular-weight DNA was prepared from whole blood with 10% SDS and phenol-chloroform (McKnight, 1978).

Typing by PCR with sequence-specific primers (PCR-SSP) and nucleotide sequencing

The first round of amplification by PCR with the locus-specific primers ERB3N and HL031 and subsequent amplification by PCR-SSP with distinct, group-specific 3' primers (Sp1 through Sp8) and a locus-specific 5' primer DRB3B were performed as described previously (Takeshima *et al.* in press). In addition, the locus-specific 3' primer, DRB3ALL, which allowed amplification of the alleles in all previously identified groups was used (Takeshima *et al.* in press).

After PCR-SSP, 5 μl of the reaction mixture was fractionated by electrophoresis on a 2% agarose/TAE gel. The gel was stained with ethidium bromide and bands of DNA were visualized under UV light.

DNA sequencing of each product of PCR-SSP was performed by the dideoxy chain termination method (Sanger *et al.* 1977) with a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an automated sequencer ABI PRISM™ 310 Genetic Analyzer (Applied

Biosystems, Foster City, CA) or with a the CEQ™2000 DNA analysis system (Beckman Coulter Inc., Fullerton, CA), as described previously (Takeshima *et al.* in press).

Sequence analysis

General sequence analysis was performed using the UWGCG program (Devereux *et al.* 1984). Nucleotide sequences were compared with sequences in the GenEMBL database using the BLAST (Gish and States 1993) algorithm.

Statistical analysis

Allele frequencies *f* were obtained by direct counting. Unbiased estimates of the expected heterozygote frequencies (*H*) given in Table 2 were computed as $n[1-{}^{k}\Sigma_{j=1}(n_j/n)^2]/(n-1)$, where n_1, n_2 and n_k are the number of k alleles at a locus in a sample of n genes drawn from breed (Edwards *et al.* 1992). The observed heterozygote frequencies (*H*) given in Table 2 were obtained directly by dividing the number of heterozygous individuals by the total number. Standard errors for alleles frequencies and heterozygosity given in Table 2 were computed as the square root of the variance of a binomial distribution $f(1-f)/2N$ and $f(1-f)/N$, where *f* represents the frequency and N the number of typed individuals (Edwards *et al.* 1992).

Pairwise genetic distance measures were estimated based on Kimura's two-parameter model (Kimura 1980). A distance tree was constructed from the distance matrix based on neighbor-joining method of Saitou and Nei (1987). In order to test the significance of the branches, 1000 bootstrap replicates were conducted (Felsenstein 1985). Breed diversity analysis based on allele frequency was conducted using the computer program gd5.exe. This program estimated

minimum distance matrix based on Nei's genetic distance (Nei 1972) and this distance tree was constructed using neighbor-joining method. Pairwise comparisons of nucleotide and amino acid substitutions between alleles were conducted according to number of difference. All of these analyses were estimated using the computer program MEGA (Kumar *et al.* 1993).

Results

Identification of BoLA-DRB3 alleles obtained from four distinct breeds of cattle

The genotypes of 419 samples from four different cattle populations from Japan were determined for *exon 2* of the *BoLA-DRB3* allele by PCR-SBT typing (Table 3-1). The 35 different previously published alleles and the three novel alleles were identified: 20 published alleles were obtained from 102 Japanese Shorthorn; 12 published alleles were obtained from 17 Jersey; 18 published alleles were obtained from 100 Holstein; and 24 alleles including 3 new alleles were obtained from 200 Japanese Black.

Figure 3-1 shows an alignment of the predicted amino acid sequence of the β1 domain encoded by all 38 *DRB3* alleles be found in this study. These alleles were 80.0% to 100% identical at the nucleotide level and 77.9% to 100% homology at the amino acid level to *BoLA-DRB3* cDNA clone NR1 (Aida *et al.* 1995). Moreover, the three new alleles were named as subtypes of alleles found in previous studies by the BoLA nomenclature committee (http://www2/ri/bbsrc/ac/uk/bola/). One new allele has been designated *DRB3*1302*, differed from *DRB3*1301* at positions 30 and 37. An other new allele was designated *m40sp6* and

differed from *BoLA-DRB3*0501* at position 86. The remaining *DRB3*4002* differed from *DRB3*4001* at positions 12, 26, 30 and 38.

Distribution of BoLA-DRB3 alleles in four natural populations of cattle

To differentiate the allelic variations between four distinct breeds, we determined the gene frequencies of the *BoLA-DRB3* alleles in each breed and compared with those of other populations (Table 3-2). All breeds examined showed extremely high *DRB3* diversity, with heterozygosity rates of between 90.5% and 100%, which were near the heterozygosity rates expected of between 90.2% to 91.3%. The *DRB3*1101* was most frequent allele in Holstein (16.7%) and was detected at moderate frequencies in Japanese Shorthorn (8.0%), Jersey (5.9%) and Japanese Black (11.0%). In contrast, the 3 alleles (*DRB3*1701, *2201* and **3202*) were detected in only Holstein (Table 3-1). The *DRB3*1001* among 24 alleles tested was most frequent allele in Japanese Black, but not frequently in Holstein (5.9%) and absent in Jersey and Japanese Shorthorn. Further studies, we established that 3 out of 5 alleles (*DRB3*0502, *1103, *1302, *4002,* and *m40sp6*) were also new alleles only in Japanese Black (Table 3-1). Within 102 Japanese Shorthorn studied, the *DRB3*1201* and *DRB3*0301* were the most and the second most common with gene frequencies of 16.0% and 14.5%, respectively. Moreover, the 5 alleles such as *DRB3*1301, *1801, *2002, *2601* and **2710* were only present in Japanese Shorthorn (Table 3-1). In spite of the low sample size about Jersey, the *DRB3*0201* and *DRB3*4501* were detected a most frequency of 17.6%. It is interesting that the *DRB*4501* was absent in other breed. Similarly, the 4 alleles (*DRB3*2006, *2801, *3701* and **2502*) were also detected only in Jersey (Table 3-1).

Phylogenetic tree based on BoLA-DRB3 sequences

Next, Neighbor-joining tree were constructed based on Kimura's two parameter distance of these 38 different *BoLA-DRB3* sequences (Fig. 3-2). The sharp of the tree clearly shows that these sequences form five major branches and the breed-specific alleles were found from four different breeds are included on same branch. Thus, it appeared that there is no clustering of sequences found in each breed of Japanese shorthorn, Japanese Black, Holstein and Jersey cattle population. This result indicated that these alleles are derived from common ancestral alleles that existed prior to the divergence of these cattle breeds.

Phylogenetic tree based on of BoLA-DRB3 allele frequencies

To clarify the evolutionary relationships among four distinct breeds, a phylogenetic tree was constructed based on the allele frequencies from *BoLA-DRB3* using the Neighbour-joining method (Fig. 3-3). The results demonstrated the shortest genetic distances for *DRB3* between the breed of Holstein and Japanese Black (0.0258). In contrast, Jersey had a large genetic distance to other three breeds. Collectively, these results suggested that Holstein and Japanese Black were the closest to each other, but Jersey was farther from these both breeds than Japanese Shorthorn.

Discussion

Recently, we developed a very precise new method for PCR-SBT which allows identifications of specific *BoLA-DRB3* alleles in large numbers of animals (Takeshima *et al.* in

press). In this study, we determined the nucleotide sequences of *exon 2* of *BoLA-DRB3* alleles of 419 samples from four different cattle populations in Japan by our new method and identified the 35 different previous published alleles and the three novel alleles. Furthermore, we demonstrate that the *BoLA-DRB3* gene is highly polymorphic in four distinct breeds and significant differences in *BoLA-DRB3* allelic frequencies between four breeds. Here, two major conclusions can be reached from the results of the allele frequencies and a Neighbor-joining tree were constructed based on these *BoLA-DRB3* sequences. First, the present study suggested that the remarkably dissimilar distribution patterns of *BoLA-DRB3* alleles in different breeds may be led by differential selection after the separation of the major population groups of cattle. Second, it appeared that the *BoLA-DRB3* sequences isolated from single breed were shared between other breeds and there is no clustering of sequences found for each breed in phylogenetic tree, suggesting that these allelic lineages are derived from common ancestral alleles that existed prior to the divergence of these cattle breeds. Moreover, by phylogenetic tree was constructed based on the allele frequencies from *BoLA-DRB3*, we clarified the evolutionary relationships among four distinct breeds: Holstein and Japanese Black were the closest to each other, but Jersey was farther from the these both breeds than Japanese Shorthorn. Thus, to our knowledge, this is the first report of the analysis of genetic background of Japanese Black, Japanese Shorthorn, Holstein and Jersey.

Most of the previous studies of the *BoLA-DRB3.2* gene have involved Holstein cows or a combination of various breeds using PCR-RFLP (Sharif *et al.* 1998; Deiz *et al.*1997a). Sharif *et al.* shows that *BoLA-DRB3.2**22* were most frequent allele in Holstein and it's value was 20.1%, next frequent allele was *BoLA-DRB3.2**24* (19.2%). Moreover, Deitz *et al.* showed that *DRB3.2**22* and **24* were most frequent alleles and these frequencies were 14.3%. In this study,

the *DRB3*1101* was most frequently allele in Holstein (16.7%) and the *DRB3*0101* had medium frequency (11.3%). The *DRB3*1101* and *DRB3*1103* were corresponding to *DRB3.2*22*, and the *DRB3*0101* were corresponding to *DRB3.2*24*. Next, few studies have analyzed exclusively the *BoLA-DRB3.2* gene of Jersey cattle (Sharif *et al.*1998 and Gilliespie *et al.* 1999). Our results confirm the data by Sharif *et al.* (1998) that the *BoLA-DRB3.2*7* was the most frequently allele (17.2%) and the data by Gilliespie *et al.* (1999) that *DRB3.2*15* was second frequently allele (13.6%). Namely, the *BoLA-DRB3*0201* and *BoLA-DRB3*4501* were most frequently alleles from Jersey cattle in this study and the PCR-RFLP types of these alleles were *BoLA-DRB3.2*7* and *BoLA-DRB3.2*15*, respectively.

In conclusion, we investigated genetic polymorphisms of the *BoLA-DRB3* genes among Japanese Black, Japanese Shorthorn, Holstein and Jersey in Japan and the phylogenetic relationship to each other. An extreme degree of genetic polymorphism in the *BoLA-DRB3* system could contribute to the understanding of the origins of a given population, and it is necessary to perform further BoLA analysis including other class II region such as DQ and class I genotyping in order to elucidate the precise genetic background of these cattle breeds. Moreover, frequencies of *BoLA-DRB3* genes in natural cattle population appeared to be extremely important to identify of a association between *DRB3* and disease.

Table 3-1
BoLA-DRB3 alleles found in four distinct cattle breeds.

Breed	No. of studied animal	Detected alleles [a, b, c]	No. of detected allele	No. of breed-specific allele	No. of new allele
Holstein	100	*0101, *0201, *0301, *0601, *0701, *0901, *0902, *1001, *1101, *1201, *14011, *1501, *1601, ***1701**, *20012, ***2201**, *2703, ***3202**	18	3	0
Japanese Shorthorn	102	*0101, *0201, *0301, *0501, *0503, *0701, *0801, *0901, *0902, *1101, *1201, ***1301**, *14011, ***1801**, *20012, ***2002**, ***2601**, *2703, ***2710**, *3401	20	5	0
Jersey	17	*0201, *0301, *0701, *0801, *1101, *1601, ***2006**, ***2801**, ***3701**, *4401, ***4501**, ***2502**	12	5	0
Japanese Black	200	*0101, *0201, *0501, ***0502**, *0503, *0701, *0801, *0901, *0902, *1001, *1101, ***1103**, *1201, ***1302**, *14011, *1501, *1601, *1801, *20012, *2703, *3401, ***4002**, *4401, **m40sp6**	24	5	3
Total	419		38	19	3

[a] DDBJ/ EMBL/ Genbank accession numbers are AB033396 for *BoLA-DRB3*1302*, AB033404 for *BoLA-DRB3*4002*

[b] Breed-specific alleles in each breeds were shown by bold character.

[c] New alleles were shown by underline.

Table 3-2
BoLA-DRB3 allele frequencies, heterozygosities (*H*) and standard error (%) in studied cattle breeds.

DRB3 allele	Holstein[a] (*N* =102) (allele no. 204)	Japanese Shorthorn[a] (*N* =100) (allele no. 200)	Jersey[a] (*N* =17) (allele no. 34)	Japanese Black[a] (*N* =200) (allele no. 400)
0101	11.3 ± 2.2	7.5 ± 1.9	0.0	3.5 ± 0.9
0201	5.4 ± 1.6	7.5 ± 1.9	**17.6 ± 6.5**	4.5 ± 1.0
0301	0.5 ± 0.5	14.5 ± 2.5	5.9 ± 4.0	0.0
0501	0.0	6.5 ± 1.7	0.0	0.5 ± 0.4
0502	0.0	0.0	0.0	3.0 ± 0.9
0503	0.0	0.5 ± 0.5	0.0	2.5 ± 0.8
0601	4.4 ± 1.4	0.0	0.0	0.0
0701	1.0 ± 0.7	0.5 ± 0.5	5.9 ± 4.0	4.8 ± 1.1
0801	0.0	12.0 ± 2.3	5.9 ± 4.0	1.0 ± 0.5
0901	1.0 ± 0.7	1.0 ± 0.7	0.0	1.8 ± 0.7
0902	5.9 ± 1.6	4.5 ± 1.5	0.0	7.3 ± 1.3
1001	5.9 ± 1.6	0.0	0.0	**17.5 ± 1.9** [b]
1101	**16.7 ± 2.6** [b]	8.0 ±1.9	5.9 ± 4.0	11.0 ± 1.6
1103	0.0	0.0	0.0	1.3 ± 0.6
1201	14.2 ± 2.4	**16.0 ± 2.6** [b]	0.0	8.5 ± 1.4
1301	0.0	1.0 ± 0.7	0.0	0.0
1302	0.0	0.0	0.0	5.0 ± 1.1
14011	4.9 ± 1.5	5.0 ± 1.5	0.0	2.0 ± 0.7
1501	13.2 ± 2.4	0.0	0.0	7.8 ± 1.3
1601	3.9 ± 1.4	0.0	2.9 ± 2.9	12.8 ± 1.7
1701	1.0 ± 0.7	0.0	0.0	0.0
1801	0.0	3.5 ± 1.3	0.0	0.0
20012	1.0 ± 0.7	3.5 ± 1.3	0.0	1.3 ± 0.6
2002	0.0	3.5 ± 1.3	0.0	0.0
2006	0.0	0.0	5.9 ± 4.0	0.0
2201	0.5 ± 0.5	0.0	0.0	0.0
2601	0.0	0.5 ± 0.5	0.0	0.0
2703	8.8 ± 2.0	1.0 ± 0.7	0.0	1.5 ± 0.6
2710	0.0	0.5 ± 0.5	0.0	0.0
2801	0.0	0.0	2.9 ± 2.9	0.0
3202	0.5 ± 0.5	0.0	0.0	0.0
3401	0.0	3.0 ± 1.2	0.0	0.3 ± 0.2
3701	0.0	0.0	8.8 ± 4.9	0.0
4002	0.0	0.0	0.0	0.8 ± 0.4
4401	0.0	0.0	5.9 ± 4.0	1.3 ± 0.6
4501	0.0	0.0	**17.6 ± 6.5** [b]	0.0
2502	0.0	0.0	14.7 ± 6.1	0.0
m40sp6	0.0	0.0	0.0	0.5 ± 0.4
$H^{observed}$	94.1	92.0	100.0	90.5
$H^{expected}$	90.2 ± 0.7	91.2 ± 0.7	91.3 ± 2.3	91.3 ± 0.5

[a] N, number of typed unrelated individuals. *H*, heterozygosity rate.

[b] bolds, the most frequent alleles in breed.

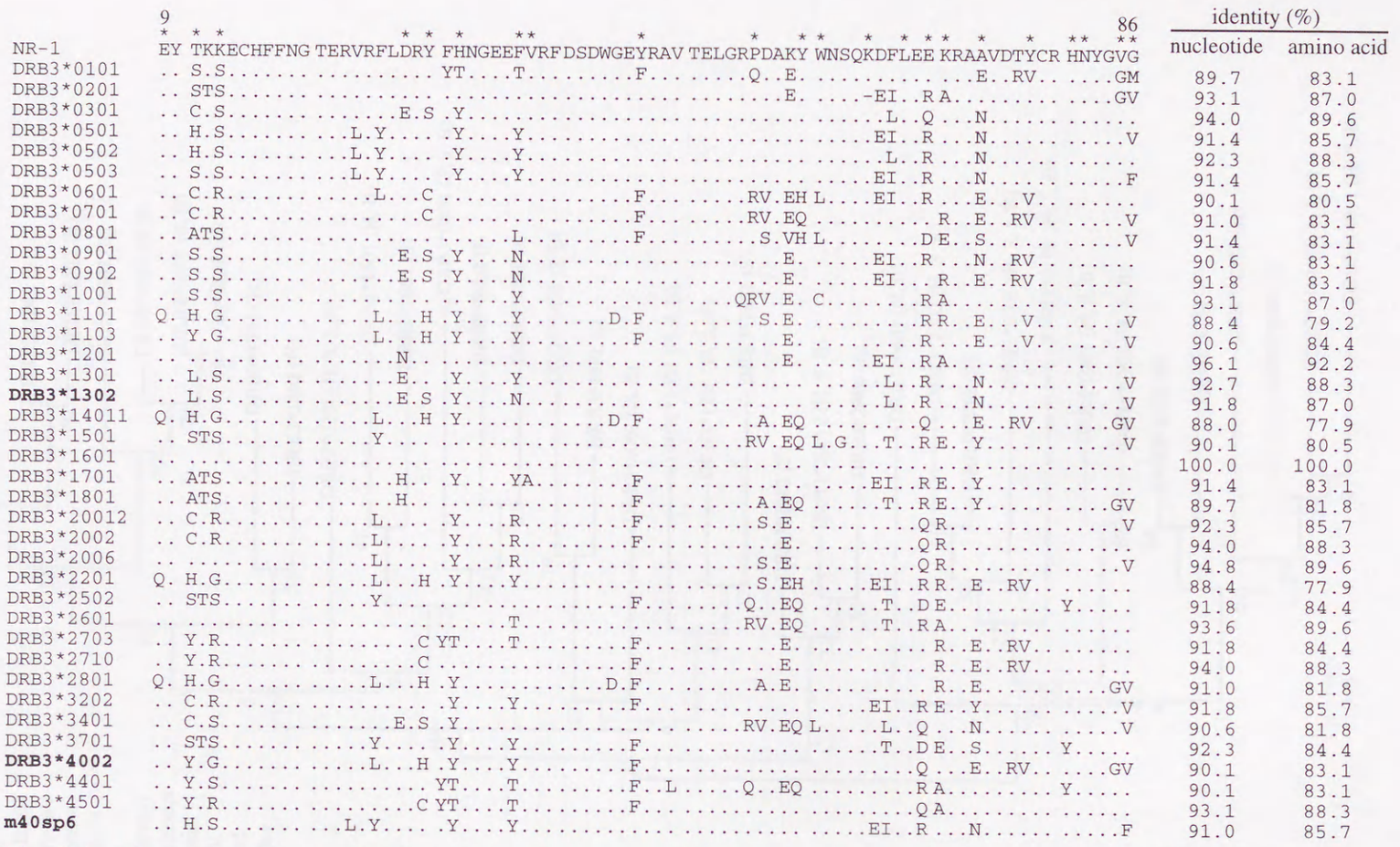


Fig. 3-1 Alignment of the predicted amino acid sequences of the β1 domain encoded by 38 *BoLA-DRB3* alleles derived from 419 animals including four distinct cattle breeds, Holstein, Japanese Shorthorn, Japanese Black and Jersey. New alleles were shown by bold character. Asterisks above residues indicate residues in the antigen recognition site in proposed model of the class II molecule (Brown et al. 1993). The numbers denote positions of amino acids in the mature protein. Nucleotide and amino acid identities were shown by comparing NR-1 *BoLA-DRB3* cDNA clone (Aida et al. 1995).

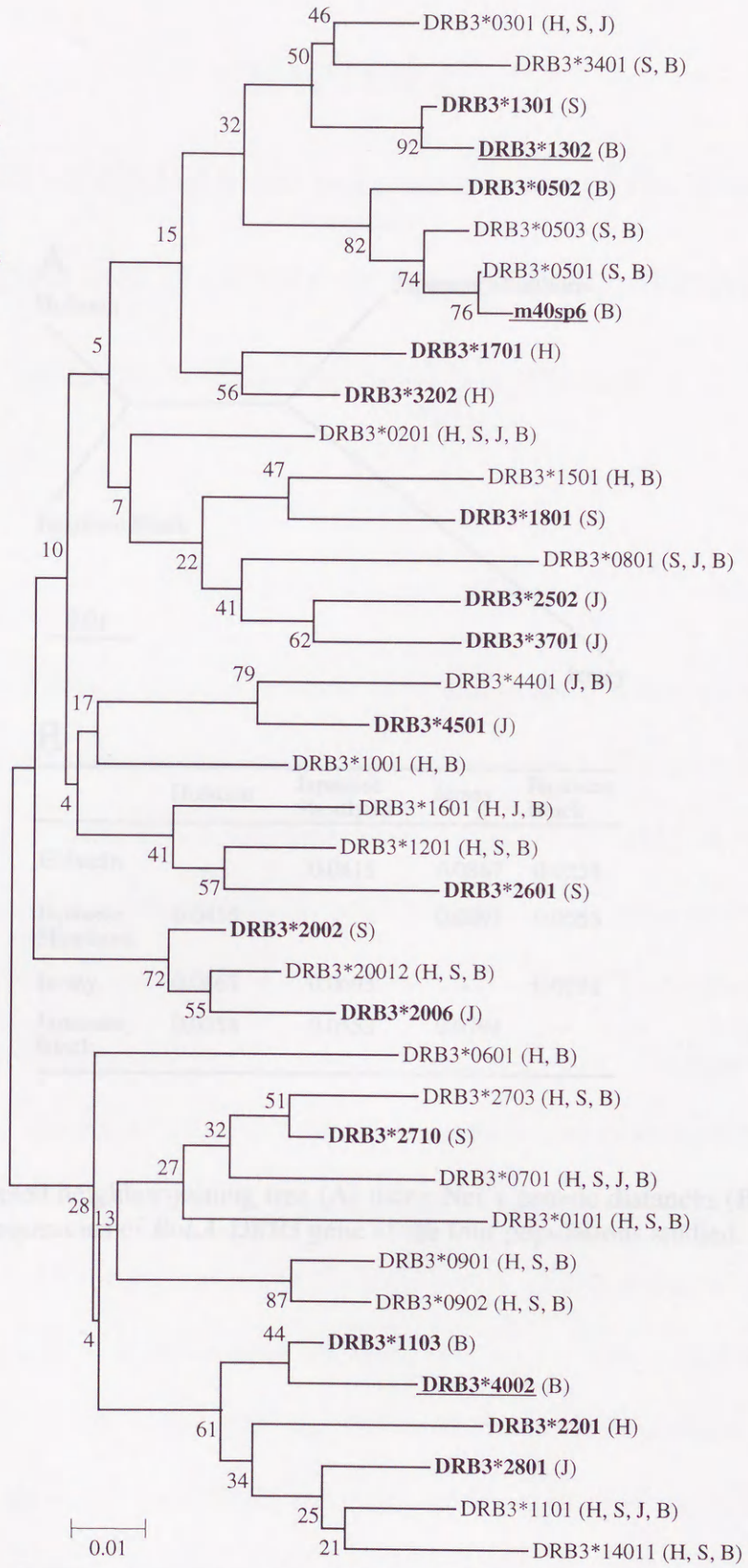
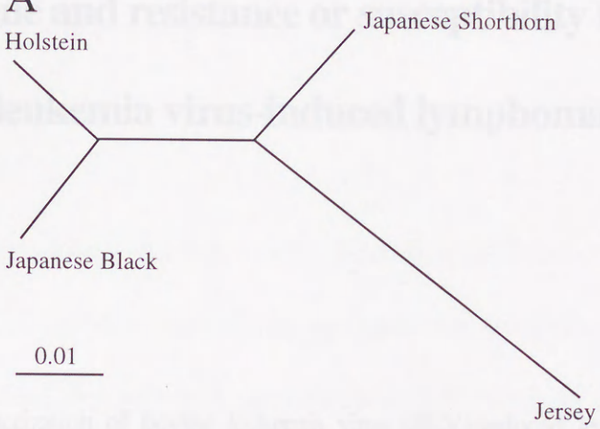


Fig. 3-2 Neighbor-joining tree using 236-bp nucleotide sequences of β1 domain coding region in *BoLA-DRB3* genes based on Kimura's two-parameter distance. Numbers are bootstrap percentages in support of each node. Bootstrapping was performed using 1000 replicates to assess the reliability of individual branches. Breed-specific alleles are shown by bold character. New alleles are shown by underline.

A



B

	Holstein	Japanese Shorthorn	Jersey	Japanese Black
Holstein	-	0.0415	0.0867	0.0258
Japanese Shorthorn	0.0415	-	0.0693	0.0553
Jersey	0.0867	0.0693	-	0.0791
Japanese Black	0.0258	0.0553	0.0791	-

Fig. 3-3 Unrooted neighbor-joining tree (A) using Nei's genetic distances (B) based on the allele frequencies of *BoLA-DRB3* gene of the four populations studied.

CHAPTER 4

The relationship between polymorphism of the *BoLA-DRB3* gene and resistance or susceptibility to bovine leukemia virus-induced lymphoma

Abstract

For the characterization of bovine leukemia virus (BLV)-induced leukemogenesis, we investigated the yet unknown association between polymorphism of *BoLA-DRB3* gene and BLV-induced lymphoma. The nucleotide sequencing of *exon 2* of the *BoLA-DRB3* gene, which is highly polymorphic, of 81 BLV-infected animals with 3 independent stages such as an aleukemic healthy, persistent lymphocytosis (PL) and lymphoma was determined by PCR-sequenced-based typing. I identified 23 distinct *BoLA-DRB3* alleles, including 3 new alleles. The population of healthy cattle positive for *BoLA-DRB3*14011* was higher than the proportion-bearing individuals in 200 control cases that were positive for the same allele. By contrast, the *BoLA-DRB3*1601* alleles was found most frequently in cattle with PL and lymphoma. Sequence analysis revealed that, approximately 56% of 43 BLV-infected but healthy cattle carry at least one *BoLA-DRB3* allele encoding Arg^{71} or Lys^{71}, Glu^{74}, Arg^{77} and Val^{78} of β1 domain of DR molecule, which suggested that alleles encoding the KERV and RERV motifs might protect against tumor development. By contrast, approximately 70% of 23 BLV-infected cattle with lymphoma carry

two alleles encoding Ala^{74}, Thr^{77} and Tyr^{78}, indicating that the ATY/ ATY genotype might be associated with susceptibility to lymphoma. Such, these results suggest that the existence of alleles associated with resistance and susceptibility to BLV-induced leukemogenesis.

Introduction

The pathogenesis of infections clearly involves immunoregulatory host factors, which include products of the MHC. The MHC molecules are glycoproteins on the surface of receptor cells that bind peptides and present them to T cells (Jorgensen *et al.* 1992, Germain and Margulies 1993). This interaction causes stimulation of T cells and activation of an immune response. Polymorphic residues in both class I and class II molecules are clustered within the peptide-binding region and are responsible for the different peptide specificities of different histocompatibility molecules. Both class I and class II molecules have allele-specific binding motifs. Peptides bound by class I molecules are of defined length (8-10 residues) (Falk *et al.* 1991). Peptides bound by class II molecules are longer with no apparent restrictions with respect to peptide length (Rudensky *et al.* 1992, Chicz *et al.* 1992). The main-chain atoms of the peptides form hydrogen bonds with MHC residues that are conserved in most class II molecules, and polymorphic pockets at the binding site accommodate the side chains. These pockets appear to determine the peptide specificity of different class II proteins (Stern *et al.*, 1994). Thus, polymorphic residues in the binding cleft for MHC class II molecules, control the binding of foreign peptides and also indirectly, the immune response to these peptides. Moreover, nucleotide sequence polymorphism in the genes for MHC class II molecules determines the specificity of the immune response and plays a role in conferring

resistance or susceptibility to chronic autoimmune disorders such as rheumatoid arthritis, insulin-dependent diabetes mellitus, pemphigus vulgaris and multiple sclerosis; to infectious diseases, such as tuberculoid leprosy and malaria; and to malignancies, such as carcinoma and melanoma. The cited studies indicate that certain class II polymorphic amino acid residues in class II molecules are strongly associated with susceptibility to disease. However, another possible explanation for association of the MHC and disease is that the association of a particular allele with a disease might be the result of so-called linkage disequilibrium between the allele and the disease-related locus.

BLV and HTLV-I and II constitute a unique subgroup within the retrovirus family that is characterized by distinct genetic content, genomic organization, and strategy for gene expression (Burny *et al.* 1988: Coffin 1996). Replication of these viruses is not only regulated by the classical structural genes of retroviruses but it is also regulated at the transcriptional level (Sodroski *et al.* 1984: Sagata *et al.* 1985) and the posttranscriptional level (Hidaka *et al.* 1988, Derse 1988) by the Tax and Rex, respectively, which are products of their own genes. BLV is associated with enzootic bovine leukosis, which is the most common neoplastic disease of cattle. Infection by BLV can remain clinically silent, with the host in an aleukemic state, or nomal B-lymphocytes and, more rarely, as B cell lymphoma in various lymph nodes after a long latent period (Burny *et al.* 1988). However, the way in which BLV induces three stages is still unknown. Infection by BLV is followed by a long latency period, and later only a certain small proportion of infected individuals rapidly develop the terminal disease. Moreover, virus expression is latent within most infected cells and circulating lymphocytes carry integrated proviral DNA but do not produce detectable virus. Therefore, infection with BLV probably is

not sufficient for leukemogenesis; some additional rare events must be involved in the leukemogenic process.

In BLV, the genes for *BoLA* appear to be important genetic determinants of resistance or sensitivity to subclinical progression of BLV infection. The *BoLA* class II genes were shown to be more strongly associated with resistance and susceptibility to PL than the *BoLA-A* locus. Among *BoLA* class II genes, *BoLA-DRB3* functional genes, which are highly polymorphic, were found to have a stronger association with susceptibility to PL than *DRB2* or *DQB*. Nucleotide sequence analysis of *exon 2* of these DRB3ALLeles revealed the presence of codons for specific amino acids at positions 70-71 of the β1 domain exclusively in alleles associated with resistance to PL (Xu *et al.*, 1993b). Thus, these results pointed strongly towards involvement of *BoLA-DRB3* in the subclinical progression of BLV infection. However, the association between development of cancer and the *BoLA* complex remains unknown. Therefore, we choused to study sheep, which provide a useful experimental model for infection by BLV and which carry MHC class II genes that are very similar to genes for BoLA, and identified *OLA-DRB1* alleles (corresponding the *BoLA-DRB3*) that were associated with resistance and with susceptibility to BLV-induced tumorigenesis (Nagaoka *et al.*, 1999). I also examined the responses to vaccination with specific peptides and a subsequent challenge with BLV in sheep that carried resistance or susceptibility alleles. Our results indicated that differences in immunoresponse were due to differences in class II alleles and reflected the risk of BLV-induced leukemogenesis.

Here, to investigated the relationship between polymorphism of *BoLA* gene and resistance or susceptibility to BLV-induced lymphoma, the nucleotide sequencing of *exon 2* of the *BoLA-DRB3*

gene of 81 BLV-infected and of control Japanese Black cattle was determined by PCR-sequenced-based typing.

Materials and Methods

Animals

Blood samples were obtained from 200 normal Japanese Black cattle, 43 BLV-infected but clinically normal cattle and15 BLV-infected cattle with lymphocytosis (Table 4-1). Tumor tissues were obtained from 23 BLV-infected cattle with lymphoma (Table 4-1). BLV-infected cattle were classified according to the previously established criteria (Aida *et al.* 1992: Levy *et al.* 1977) and the genomic integration of BLV provirus.

DNA extraction

Total chromosomal DNA was extracted from PBL as described by Hughes *et al.* (1978) and from frozen blocks of tumor tissue with 10% SDS and phenol-chloroform (McKnight 1978).

Typing by PCR with sequence-specific primers (PCR-SSP) and nucleotide sequencing

The first round of amplification by PCR was performed with the locus-specific primers ERB3N (Takeshima *et al.*, in press) and HL031 (van Eijk *et al.* 1992) and subsequent amplification by PCR-SSP was performed with distinct, group-specific 3' primers (Sp1 through Sp8) (Takeshima *et al.*, in press) and a locus-specific 5' primer DRB3B (Takeshima *et al.*, in press). In addition, the

locus-specific 3' primer, DRB3ALL, which allowed amplification of the alleles in all previously identified groups was used (Takeshima *et al.,* in press).

Reactions performed with 20 ng of DNA (1 μl) in a final volume of 50 μl. The reaction mixture contained 49 μl of PCR buffer [final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 0.1% TritonX-100], with 120 μM dNTPs, 1.5 mM $MgCl_2$, 200 μM each primer and 1 unit of recombinant rTaq DNA polymerase (Toyobo co., Ltd., Osaka, JP). The thermal cycling profile for the first round of amplification included an initial denaturation for 5 min at 95℃, which was followed by 20 cycles of 50 sec at 95℃, 50 sec at 60℃ and 50 sec at 72℃. The final extension reaction was allowed to proceed for 10 min at 72℃. Subsequently, 1-μl aliquots of the mixture after the first PCR were transferred to cells of a 96-well plate. Each well contained 24 μl of GeneAmp^R Gold Buffer [final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% TritonX-100] plus 120 μM dNTPs, 1.5 mM $MgCl_2$, 400 μM each primer and 1 unit of Taq polymerase (AmpliTaq^{TM} Gold, Applied Biosystems, Foster City, CA) for a PCR-SSP with the following thermal cycling profile: initial denaturation for 10 min at 95℃, followed by 20 cycles of 1 min at 95℃, 30 sec at 64℃ and 30 sec at 72℃, with final extension for 5 min at 72℃. All reactions were performed in a TGRADIENT Thermocycler 96 (Biometra, Goettingen, DE).

After PCR-SSP, 5 μl of the reaction mixture were fractionated by electrophoresis on a 2% agarose/TAE gel. The gel was stained with ethidium bromide and bands of DNA were visualized under UV light.

A CEQ^{TM}2000 Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., Fullerton, CA) was used to sequence each product of PCR-SSP with a -21-mer M13 universal primer, 5'-TGT AAA ACG ACG GCC AGT-3' or an M13 reverse primer, 5'-CAG GAA ACA GCT ATG

ACC-3'. I combined 6 μl of the reaction mixture after the second-round PCR, which had been diluted 10-fold by addition of distilled water, 1 μl of a solution of 12 pmol/μl sequencing primer, 2 μl of 10x Sequencing Reaction Buffer, 1 μl of dNTP Mix, 2 μl of ddUTP Dye Terminator, 2 μl of ddGTP Dye terminator, 2 μl of ddCTP Dye Terminator, 2 μl of ddATP Dye Terminator, and 1 μl of the solution of DNA polymerase. Then cycle sequencing reactions were performed as described in the manual from the manufacturer of the kit. Reaction mixtures were applied to the CEQ™ 2000 DNA analysis system (Beckman Coulter Inc.). Data were collected with the software package provided with the CEQ™2000 sequencing system. In addition, sequencing of part of each product of PCR was performed with a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) as described previously (Takeshima *et al.,* in press).

Analysis of data

Raw data after chromatography were collected with Sequencing Analysis™ and Factura™ programs (Applied Biosystems). Nucleotide sequences were compared with sequences in the GenEMBL database using the BLAST (Gish and States 1993) algorithm.

PCR-RFLP

The *DRB3 exon 2* fragment was amplified firstly by the PCR using the locus-specific primers ERB3 (Nagaoka *et al.* 1999) and HL031 (van Eijk *et al.* 1992) and then it was amplified with the locus-specific primers ERB3 and SRB3 (Nagaoka *et al.* 1999). Each product of PCR was incubated for 4 h at 37℃ with three units of *Rsa*I or *Hae*III in a final volume of 20 μl, or for 1

h at 60℃ with three units of *Bst*YI in a total of 20 μl. Restriction fragment patterns were examined after electrophoresis on a 12% polyacrylamide gel or a 5% agarose/TAE gel.

Statistical methods

The results were statistically analyzed using SAS software.

Other Procedures

BLV provirus integrated into the host's genome was amplified from chromosomal DNA (100 ng to 1mg) by PCR with two sets of primers : (a) 7192 and 533; and (b) 7192 and 8580 (52). For detection of antibodies against BLV, an immunodiffusion test was performed with BLV gp51 and internal protein 24 as antigens.

Results

Identification of BoLA-DRB3 alleles from BLV-infected cattle

The 81 BLV-infected cattle (Japanese Black breed) were classified into three stages, according to serological detection, clinical signs, histological observation, total number of lymphocytes in blood, and genomic integration of BLV provirus (Table 4-1). Firstly, we determined the nucleotide sequences of *exon 2* of the *BoLA-DRB3* gene in each animal by PCR-SBT method (Table 4-1) and checked the sequence of the alleles from an individual against the PCR-RFLP genotype (Table 4-1). Next, we compared the deduced amino acid sequence of the β1 domain encoded by these alleles with those sequence encoded by 102 previously reported

BoLA-DRB3 alleles (Groenen *et al.* 1990; Davies *et al.* 1994a; Russell *et al.* 1997; Takeshima *et al.*, in press; http://www2.ri.bbsrc.ac.uk/bola/) and identified 23 distinct alleles.

Frequency of BoLA-DRB3 alleles in BLV-infected cattle at different stages

The numbers of individuals that were positive for each of the different *BoLA-DRB3* alleles in cattle at the asymptomatic stage, PL and the lymphoma stage, and control cases are shown in Table 4-2. The population of healthy cattle positive for *BoLA-DRB*14011* was higher than the proportion-bearing individuals with lymphoma that were positive for the same. By contrast, the*BoLA-DRB3*1601* allele was found most frequently in cattle with lymphoma. No other alleles were associated with a statistically significant difference between the two populations. This result suggested the presence of epitope(s) encoded by the the*BoLA-DRB3*1601* and **14011* sequence that showed be important for disease susceptibility or resistance.

Association of BoLA-DRB3 epitopes with BLV-infected cattle

To identify the amino acid residues that were associated with sensitivity to BLV-induced lymphoma, we aligned the residues that differed among the *BoLA-DRB3* alleles that were positively and negatively associated with tumor progression (Fig. 4-1). The *BoLA-DRB3*1601* allele appeared to encode amino acids KATY at positions 71, 74, 77 and 78 of β1 domain. By contrast, the *BoLA-DRB3*14011* allele encode amino acids RETV.

Since these residues are in an α-helixed region and point into the antigen-binding grove in the predicted model of the class II β-chain, we reexamined whether these amino acid residues might be correlated with resistance and susceptibility to lymphoma at the population level (Table 4-3).

Alleles characterized by the KERV and RERV at positions at positions 71, 74, 77 and 78 was present at a significantly higher frequency in healthy cattle as compared with cattle with lymphoma. As shown in Fig. 1, those alleles included *BoLA-DRB3*0101, BoLA-DRB3*14011, BoLA-DRB3*0701, BoLA-DRB3*0902* and *BoLA-DRB3*2703.* These results suggested that *BoLA-DRB3* alleles that encode the KERV and RERV at positions 71, 74, 77 and 78 might have a strong protective effect against BLV-induced tumor development. By contrast, alleles characterized by the KATY and AATY at positions 71, 74, 77 and 78 in lymphoma cases were present of a statistically significant elevated frequency in lymphoma cases as compared with healthy. These results demonstrated that *BoLA-DRB3* alleles that encoded the KATY and AATY at positions at positions 71, 74, 77 and 78 such as *BoLA-DRB3*1601, *0201, *1001, *1201, *4401*were positively related to BLV-induced tumor development.

Next, we compared frequencies of *BoLA-DRB3* genotypes at the 3 stages of the disease (Table 4-4). In cattle with lymphoma, approximately 70% were homozygous for *DRB3* alleles encoding ATY of β1 domain, indicating that the ATY/ATY genotype might be associated with susceptibility to lymphoma. By contrast, in BLV-infected but healthy cattle, approximately 56% of BLV-infected but healthy cattle carry at least one *BoLA-DRB3* allele encoding KERV and RERV of β1 domain of DR molecule, which suggested that alleles encoding the KERV and RERV motifs might protect against tumor development.

Discussion

These results indicate the existence of alleles associated with resistance and susceptibility to BLV-induced leukemogenesis. In addition, it appears that susceptibility to tumor development might be determined by polymorphic residues binding to antigenic peptides directly within the binding cleft of the MHC-DR molecule (Brown *et al.*1993). Since infection with BLV probably is not sufficient for leukemogenesis; some additional rare events, for example, a mutation in the p53 tumor-suppressor (Tajima *et al.* 1998), must be involved in the leukemogenic process, class II genes may be important host-related Immunogenetic factors in the susceptibility to BLV-induced lymphoma.

In previous study, Xu *et al.* shows that resistance-associated DRB3 alleles share a ER motif, at positions 70-71, and susceptibility-associated DRB3 alleles most often had VDTY or VDTY at positions 75-78, with PL in Holstein cattle (Xu *et al.* 1993b). In this study, we described that resistance-associated motif with leukemia were KERV/RERV, at position 71,74,77 and 78. ER motif which Xu *et al.* described were part of this but different alleles were included in KERV/RERV motif. Thus, BLV-associated PL and lymphoma had the different factor of association with *BoLA-DRB3*. Zanotti *et al.* described about association of BoLA-class II haplotypes with subclinical progression of PL, in Holstein cattle (Zanotti *et al.* 1996.). They show that DQA*3A-DQB*3A-DRB2*2A-DRB3.2*11 was resistant-associated haplotype and DQA*12-DQB*12-DRB2*3A-DRB3.2*8 was susceptible-associated haplotype with PL. Association with lymphoma in Japanese Black, which we described, was not correspond with these data. In our result, lymphoma-resistant allele was DRB3*14011 (DRB3.2*27) and Lymphoma-

susceptible allele was DRB3*1601(DRB3.2*10). Moreover, our data shows that high-risk alleles for lymphoma tend to high-risk with PL. So, the reason of these differences was because of deferential of breeds.

Additional studies are required to define in detail the mechanism of the association between susceptibility to leukemogenesis in response to BLV and polymorphism of MHC class II alleles. It has been postulated that the true disease-sensitivity gene is located near the MHC genes; therefore, MHC alleles may only be the marker genes (Rukstalis 1989). From previous study (Nagaoka *et al.* 1999), however, it seems likely that quantitative details of the immune reaction caused by particular allelic forms of MHC may contribute to the induction and maintenance of an efficient antitumor immune response and to the progression toward full-blown disease. By contrast, although the host's genetic background, such as the HLA class II alleles, seems to be an important factor in determining whether carriers of HTLV-I develop either adult T cell leukemia/lymphoma or HTLV-I-associated myelopathy, there seems to be no significant difference in frequencies of class II alleles between patients with adult T cell leukemia/lymphoma and carriers of HTLV-I (Manns *et al.* 1998). Therefore, additional studies focusing on the amino acid residues that line the pocket that accommodates the side chain of bound peptide may be useful in the case of HTLV-I. Aida *et al.* (1992; 1993) obtained evidence to suggest that a tumor-associated membrane glycoprotein that is serine-phosphorylated only during the leukemic stage of BLV-induced lymphoma is related to the BoLA-DR molecule. Therefore, alterations in the biochemical nature and physiological function of DR molecules in the progression of BLV-induced lymphoma, in association with resistance or susceptibility to tumor development, are also worthy of further examination.

Table 4-1
Characterization of Japanese Black cattle infected with BLV.

Group and animal	Age (yr)	WBC/ mm^3	PBL[a]/ mm^3	AtMC/ mm^3	Antibody against BLV	BLV provirus[b]	*BoLA-DRB3* alleles[c] (A);(B)	PCR-RFLP (A);(B)
BLV-infected but healty cattle								
T13	18	5,400	2,646	0		+	BoLA-DRB3*1201; *1501	08 /16
T19	17	6,000	1,980	120		+	BoLA-DRB3*0503; *1101	01 /22
T29	17	10,000	6,200	0		+	BoLA-DRB3*0503; *1101	01 /22
GH1	17	3,300	1,353	0		+	BoLA-DRB3*1001; *1601	03 /10
OK8	17	7,500	4,200	0		+	BoLA-DRB3*1302; *1601	02 /10
O25-Y	17	8,600	6,622	0		+	BoLA-DRB3*0701; *1601	28 /10
A16-202	16	5,300	4,187	106	(+,-)	+	BoLA-DRB3*0902; *1601	11 /10
T25	15	8,000	2,640	0		+	BoLA-DRB3*0701; *1101	28 /22
T23	14	9,100	4,459	0		+	BoLA-DRB3*0201; *0503	07 /01
A21-216	14	2,900	1,796	0	(+,-)	+	BoLA-DRB3*1501; *1601	16 /10
A22-217	14	3,400	1,326	0	(+,-)	+	BoLA-DRB3*0701; *0902	28 /11
A63-266	14	5,100	4,182	0	(+,-)	+	BoLA-DRB3*1201; *1302	08 /09
A54-254	14	4,300	3,483	0	(+,-)	+	BoLA-DRB3*0501; *1302	01 /09
YS4	14	6,200	2,790	0		+	BoLA-DRB3*1001; *2703	03 /23
GH9	14	5,200	3,640	0		+	BoLA-DRB3*1001; *1501	03 /16
A23-218	13	3,700	2,146	111	(+,-)	+	BoLA-DRB3*14011;*1501	27 /16
A28-223	13	5,400	2,700	54	(+,-)	+	BoLA-DRB3*0701;*14011	28 /27
A30-225	13	4,200	2,142	0	(+,-)	+	BoLA-DRB3*0902; *1001	11 /03
T17	13	6,500	2,470	0		+	BoLA-DRB3*0201; *1101	07 /22
GH11	13	5,800	NT	NT		+	BoLA-DRB3*1601;*20012	10 /15
S1	12	12,600	4,032		(+,-)	+	BoLA-DRB3*0201; *1401	07 /27
T11	12	7,500	3,675	150		+	BoLA-DRB3*1201; *1501	08 /16
YS1	12	6,200	4,588	0		+	BoLA-DRB3*0101; *1501	24 /16
A36-234	11	5,300	2,014	0	(+,-)	+	BoLA-DRB3*0701; *1302	28 /09
A37-235	11	5,200	3,900	0	(+,-)	+	BoLA-DRB3*14011;*14011	27 /27
A43-241	11	3,400	1,802	0	(+,-)	+	BoLA-DRB3*0902; *1001	11 /03
A42-240	11	2,500	2,275	50	(+,-)	+	BoLA-DRB3*1201;*14011	08 /27
A40-238	11	8,300	5,561	249	(+,-)	+	BoLA-DRB3*0101; *1601	24 /10
A46-245	11	5,200	4,680	104	(+,±)	+	BoLA-DRB3*1001; *1601	03 /10
T22	11	7,400	3,034	0		+	BoLA-DRB3*0701; *1501	28 /16
GH8	11	6,300	5,355	0		+	BoLA-DRB3*0701; *1302	28 /09
A51-250	10	9,500	5,605	95	(+,-)	+	BoLA-DRB3*1501; *1601	16 /10
A50-249	10	8,000	6,080	0	(+,-)	+	BoLA-DRB3*0902; *1601	11 /10
A52-252	10	3,400	3,128	0	(±,-)	+	BoLA-DRB3*1001; *3401	03 /45
T09	10	7,200	4,104	0		+	BoLA-DRB3*1101; *1201	22 /08
T14	10	6,000	3,960	60		+	BoLA-DRB3*0201; *1101	07 /22
GH10	10	4,500	3,555	0		+	BoLA-DRB3*1601; *2703	10 /23
A60-262	9	3,900	2,847	117	(+,-)	+	BoLA-DRB3*0101; *1302	24 /09
A68-271	9	5,200	10,089	0	(+,-)	+	BoLA-DRB3*1302;*14011	09 /27
T02	9	10,100	3,030	0		+	BoLA-DRB3*1001; *1201	03 /08
YS2	9	5,600	3,416	6		+	BoLA-DRB3*1001; *1601	03 /10
YS3	9	6,500	4,355	4		+	BoLA-DRB3*0101; *0502	24 /41
GH6	9	5,200	3,796	0		+	BoLA-DRB3*1001; *1601	03 /10
BLV-infected with persistent lynphoytosis (PL)								
O31-M	17	15,100	12,231	0		+	BoLA-DRB3*1201; *1501	08 /16
O27-A	17	21,800	18,530	0		+	BoLA-DRB3*1501; *1601	16 /10
S115	16	8,700	8,448		(+,-)	+	BoLA-DRB3*1001; *1601	03 /10
GH3	16	7,400	6,142	0		+	BoLA-DRB3*0801; *1001	21 /03
O195-S	16	19,100	16,426	0		+	BoLA-DRB3*1601; *1601	10 /10
GH2	15	7,700	5,929	0		+	BoLA-DRB3*1001;*m40sp6	03 /01
O197-I	15	11,500	9,315	0		+	BoLA-DRB3*1101; *1601	22 /10
O119-F	15	13,700	7,672	274		+	BoLA-DRB3*1501; *1601	16 /10
S154	13	12,300	11,377		(+,-)	+	BoLA-DRB3*0701; *1601	28 /10
OK4	12	8,300	7,221	0		+	BoLA-DRB3*1501; *1601	16 /10
T16	11	13,900	8,479			+	BoLA-DRB3*0503; *1501	01 /16
GH4	11	9,900	7,722	0		+	BoLA-DRB3*1601; *1601	10 /10
GH5	10	10,900	8,175	0		+	BoLA-DRB3*1601; *1601	10 /10
43-Y	9	16,500	9,323	0	(+,-)	+	BoLA-DRB3*0201; *1601	07 /10
A69-272	9	17,700	10,089	3,009	(+,-)	+	BoLA-DRB3*1302; *1601	09 /10
BLV-infected with lymphoma								
pr2284	20	11,000	7,370	1,100	(+,-)	+	BoLA-DRB3*0902; *1001	11 /03
GH13	16	9,200	NT	NT	NT	+	BoLA-DRB3*0201; *1601	07 /10
pr2261	15	11,200	3,248	3,696	NT	+	BoLA-DRB3*1001; *1601	03 /10
pr2476	14	31,400	10,990	14,758	(+,-)	+	BoLA-DRB3*0201; *4401	07 /20
pr1714	13	5,500	2,338	2,173	(+)	+	BoLA-DRB3*1001; *1101	03 /22
pr1717	13	12,100	5,324	1,331	(+)	+	BoLA-DRB3*1001; *1101	03 /22
pr2175	12	20,500	5,115	3,100	(+,-)	+	BoLA-DRB3*1001; *1601	03 /10
pr1720	11	5,800	4,582	203	(+)	+	BoLA-DRB3*1001; *1001	03 /03
pr2230	11	63,400	10,778	44,380	(+,-)	+	BoLA-DRB3*1601; *1601	10 /10
pr2365	11	7,100	2,201	1,775	(+,+)	+	BoLA-DRB3*0201; *1001	07 /03
pr2408	11	19,800	8,514	10,098	(+,+)	+	BoLA-DRB3*1601;*20012	10 /15
pr1693	10	9,500	2,613	380	(+)	+	BoLA-DRB3*1601;*20012	10 /15
pr2169	10	85,200	5,538	75,828	(+,-)	+	BoLA-DRB3*1601;*20012	10 /15
pr2121	10	5,200	1,560	39	(+,-)	+	BoLA-DRB3*1001; *1601	03 /10
pr2506	10	138,400	NT	129,404	(+,+)	+	BoLA-DRB3*0902; *3401	11 /45
pr2266	8	9,600	1,392	1,152	NT	+	BoLA-DRB3*1201; *1601	08 /10
pr2374	8	36,800	11,040	24,288	(++,++)	+	BoLA-DRB3*1501; *1601	16 /10
pr2487	8	15,000	5,550	1,500	(+,+)	+	BoLA-DRB3*0201; *1601	07 /10
pr2107	7	12,900	5,225	4,257	(+)	+	BoLA-DRB3*1101; *1601	22 /10
pr2297	7	27,500	12,375	6,325	(+,-)	+	BoLA-DRB3*0201; *1001	07 /03
pr2436	7	28,600	3,432	19,448	(+,-)	+	BoLA-DRB3*1601; *1601	10 /10
pr2224	6	14,100	3,807	8,813		+	BoLA-DRB3*1001; *1601	03 /10
pr2493	1	74,500	10,430	61,835	(+,+)	+	BoLA-DRB3*0501; *1501	01 /16

[a]PBL, peripheral blood lymphocytes; AtMC, artypical mononuclear cells; NT, not tested; (+), positive for detection of BLV provirus integrated in chromosomal genome DNA by PCR with a BLV-specific primer.

[b]BLV provirus was detected by PCR amplification of BLV-LTR gene.

[c]Both alleles, A and B, of each animal are shown.

Table 4-2
BoLA-DRB3 typing of nomal cattle and cattle with BLV-induced lymphoma, PL and healthy.

Allele (DRB3*)	Control cattle (n=400)		BLV-infected cattle with healthy (n=86)		PL (n=30)		lymphoma (n=46)		P values[b] (vs.control)		
	n[a]	%	n	%	n	%	n	%	Healthy	PL	Lymphoma
0101	14	3.5	4	4.7	0	0.0	0	0.0	0.539	0.612	0.379
0201	18	4.5	4	4.7	1	3.3	5	10.9	1.000	1.000	0.076
0501	2	0.5	1	1.2	0	0.0	1	2.2	0.443	1.000	0.279
0502	12	3.0	1	1.2	0	0.0	0	0.0	0.481	1.000	0.622
0503	10	2.5	3	3.5	1	3.3	0	0.0	0.710	0.553	0.609
0701	19	4.8	7	8.1	1	3.3	0	0.0	0.195	1.000	0.242
0801	4	1.0	0	0.0	1	3.3	0	0.0	1.000	0.305	1.000
0901	7	1.8	0	0.0	0	0.0	0	0.0	0.613	1.000	1.000
0902	29	7.3	5	5.8	0	0.0	2	4.3	0.817	0.280	0.758
1001	70	17.5	10	11.6	3	10.0	11	23.9	0.203	0.448	0.312
1101	44	11.0	6	7.0	1	3.3	3	6.5	0.330	0.347	0.453
1201	34	8.5	6	7.0	1	3.3	1	2.2	0.829	0.495	0.240
1302	20	5.0	7	8.1	1	3.3	0	0.0	0.295	1.000	0.248
14011	8	2.0	7	8.1	0	0.0	0	0.0	8.21E-03	1.000	1.000
1501	31	7.8	8	9.3	5	16.7	2	4.3	0.661	0.094	0.560
1601	51	12.8	13	15.1	14	46.7	16	34.8	0.598	1.81E-05	3.26E-04
20012	5	1.3	1	1.2	0	0.0	3	6.5	1.000	1.000	0.040
2703	6	1.5	2	2.3	0	0.0	0	0.0	0.636	1.000	1.000
3401	1	0.3	1	1.2	0	0.0	1	2.2	0.323	1.000	0.196
4002	3	0.8	0	0.0	0	0.0	0	0.0	1.000	1.000	1.000
m40sp6	2	0.5	0	0.0	1	3.3	0	0.0	1.000	0.070	1.000
4401	5	1.3	0	0.0	0	0.0	1	2.2	0.592	1.000	0.482
1103	5	1.3	0	0.0	0	0.0	0	0.0	0.592	1.000	1.000

[a]Number of individuals positive for a particular *DRB3* allele.
[b]Fisher's exact text was used to determine statistical significance.

Table 4-3
Association of *DRB3* epitopes with BLV-induced cattle lymphoma

Epitope	BLV-infected cattle with healthy (n=86)		PL(n=30)		lymphoma(n=46)		P values[a] (vs.healthy)	
	n	%	n	%	n	%	PL	lymphoma
V^{78}	31[b]	36.0	2	6.7	5	10.9	0.00187	0.00195
Non-V^{78} (Y^{78})	55	64.0	28	93.3	41	89.1	0.00187	0.00195
R^{77}	25	29.1	1	3.3	2	4.3	0.00223	0.000535
Non-R^{77} (T^{77})	61	70.9	29	96.7	44	95.7	0.00223	0.000535
E^{74}	31	36.0	2	6.7	5	10.9	0.00187	0.00195
Non-E74 (N^{74})	13	15.1	3	10.0	2	4.3	0.759	0.085
(A^{74})	34	39.5	19	63.3	37	80.4	0.033	0.00000791
(Y^{74})	8	9.3	5	16.7	2	4.3	0.316	0.493
(S^{74})	0	0.0	1	3.3	0	0.0	0.259	-
K^{71}	37	43.0	17	56.7	18	39.1	0.210	0.714
Non-K^{71}(R^{71})	21	24.4	2	6.7	8	17.4	0.037	0.387
(A^{71})	20	23.3	5	16.7	18	39.1	0.608	0.070
(E^{71})	8	9.3	6	20.0	2	4.3	0.189	0.493
$R^{77}V^{78}$	25	29.1	1	3.3	2	4.3	0.00223	0.000535
$T^{77}V^{78}$	6	7.0	1	3.3	3	6.5	0.675	1.000
$T^{77}Y^{78}$	55	64.0	28	93.3	41	89.1	0.00187	0.00195
$E^{74}R^{77}V^{78}$	25	29.1	1	3.3	2	4.3	0.00223	0.000535
$E^{74}T^{77}V^{78}$	6	7.0	1	3.3	3	6.5	0.675	1.000
$A^{74}T^{77}Y^{78}$	34	39.5	19	63.3	37	80.4	0.033	0.00000791
$Y^{74}T^{77}Y^{78}$	8	9.3	5	16.7	2	4.3	0.316	0.493
$N^{74}T^{77}Y^{78}$	13	15.1	3	10.0	2	4.3	0.759	0.085
$S^{74}T^{77}Y^{78}$	0	0.0	1	3.3	0	0.0	0.259	-
$K^{71}E^{74}R^{77}V^{78}$	11	12.8	0	0.0	0	0.0	0.064	0.00834
$R^{71}E^{74}R^{77}V^{78}$	14	16.3	1	3.3	2	4.3	0.111	0.053
$R^{71}E^{74}T^{77}V^{78}$	6	7.0	1	3.3	3	6.5	0.675	1.000
$A^{71}A^{74}T^{77}Y^{78}$	20	23.3	5	16.7	18	39.1	0.608	0.070
$K^{71}A^{74}T^{77}Y^{78}$	13	15.1	14	46.7	16	34.8	0.000902	0.014
$R^{71}A^{74}T^{77}Y^{78}$	1	1.2	0	0	3	6.5	1.000	0.122
$E^{71}Y^{74}T^{77}Y^{78}$	8	9.3	5	16.7	2	4.3	0.316	0.493
$K^{71}N^{74}T^{77}Y^{78}$	13	15.1	3	10.0	2	4.3	0.759	0.085
$E^{71}S^{74}T^{77}Y^{78}$	0	0.0	1	3.3	0	0.0	0.259	-

[a]Fisher's exact test used to determine statistical significance.
[b]Number of alleles positive that encoded a particular epitope at position β^{71}, β^{74}, β^{77} and/or β^{78}.

Table 4-4
Genotype frequencies based on amino acid motifs at positions $\beta^{71\ 74\ 77\ and\ 78}$

Genotype	BLV-infected cattle with healthy (n=43)		PL (n=15)		lymphoma (n=23)		P values[a] (vs. healthy)	
	n	%	n	%	n	%	PL	lymphoma
RV/RV or non-RV	22[b]	51.2	1	6.7	2	8.7	2.22.E-03	5.25.E-04
TV/TV or non-TV	6	14.0	1	6.7	3	13.0	0.664	1.000
TY/TY or non-TY	39	90.7	15	100.0	23	100.0	0.564	0.289
ERV/ERV or Non-ERV	22	51.2	2	13.3	2	8.7	0.014	5.25E-04
ERV/Non-ERV	19	44.2	2	13.3	2	8.7	0.059	4.73E-03
ETV/ETV or Non-ETV	6	14.0	0	0	3	13.0	0.323	1.000
ATY/ATY or Non-ATY	28	65.1	14	93.3	21	91.3	0.046	0.036
ATY/Non-ATY	22	51.2	10	66.7	5	21.7	0.374	0.034
NTY/NTY or Non-NTY	12	27.9	3	20.0	2	8.7	0.736	0.113
YTY/YTY or Non-YTY	8	18.6	5	33.3	2	8.7	0.288	0.474
STY/STY or Non-STY	0	0	1	6.7	0	0	0.259	-
ERV/ERV	3	7.0	0	0	0	0	0.561	0.546
ERV/ATY	10	23.3	2	13.3	1	4.3	0.712	0.081
ERV/NTY	5	11.6	0	0	1	4.3	0.313	0.656
ERV/YTY	3	7.0	0	0	0	0	0.561	0.546
ERV/ETV	1	2.3	0	0	0	0	1.000	1.000
ETV/ATY	3	7.0	0	0	3	13.0	0.561	0.413
ETV/NTY	2	4.7	0	0	0	0	1.000	0.539
ATY/ATY	6	14.0	5	33.3	16	69.6	0.131	1.45E-05
ATY/NTY	4	9.3	2	13.3	0	0	0.643	0.289
ATY/YTY	5	11.6	4	26.7	1	4.3	0.218	0.656
ATY/STY	0	0	1	6.7	0	0	0.259	-
NTY/NTY	1	2.3	0	0	0	0	1.000	1.000
NTY/YTY	0	0	1	6.7	1	4.3	0.259	0.656
KERV/KERV or non-KERV	10	23.3	0	0	0	0	0.050	0.011
KERV/ non-KERV	9	20.9	0	0	0	0	0.094	0.022
RERV/RERV or Non-RERV	13	30.2	1	6.7	2	8.7	0.087	0.065
RERV/ Non-RERV	12	27.9	1	6.7	2	8.7	0.150	0.113
RETV/RETV or Non-RETV	6	14.0	1	6.7	3	13.0	0.664	1.000
KATY/KATY or Non-KATY	13	30.2	11	73.3	14	60.9	5.67E-03	0.020
KATY/ Non-KATY	13	30.2	8	53.3	12	52.2	0.129	0.111
AATY/AATY or Non-AATY	19	44.2	5	33.3	14	60.9	0.552	0.301
AATY/ Non-AATY	18	41.9	5	33.3	10	43.5	0.760	1.000
KNTY/KNTY or Non-KNTY	12	27.9	3	20.0	1	4.3	0.736	0.025
EYTY/EYTY or Non-EYTY	7	16.3	5	33.3	2	8.7	0.265	0.478
KERV/KERV	1	2.3	0	0	0	0	1.000	1.000
KERV/RERV	1	2.3	0	0	0	0	1.000	1.000
KERV/AATY	2	4.7	0	0	0	0	1.000	0.539
KERV/KNTY	3	7.0	0	0	0	0	0.561	0.546
KERV/EYTY	2	4.7	0	0	0	0	1.000	1.000
KERV/KATY	1	2.3	0	0	0	0	1.000	1.000
RERV/RERV	1	2.3	0	0	0	0	1.000	1.000
RERV/RETV	1	2.3	0	0	0	0	1.000	1.000
RERV/KATY	4	9.3	1	6.7	0	0	1.000	0.289
RERV/AATY	3	7.0	0	0	1	4.3	0.561	1.000
RERV/KNTY	2	4.7	0	0	1	4.3	1.000	1.000
RERV/EYTY	1	2.3	0	0	0	0	1.000	1.000
RETV/KATY	0	0	1	6.7	1	4.3	0.259	0.348
RETV/AATY	3	7.0	0	0	2	8.7	0.561	1.000
RETV/KNTY	2	4.7	0	0	0	0	1.000	1.000
KATY/KATY	0	0	3	20.0	2	8.7	0.015	0.118
KATY/RATY	1	2.3	0	0	3	13.0	1.000	0.118
KATY/AATY	4	9.3	2	13.3	7	30.4	0.643	0.040
KATY/KNTY	1	2.3	1	6.7	0	0	0.454	1.000
KATY/EYTY	2	4.7	3	20.0	1	4.3	0.103	1.000
AATY/AATY	1	2.3	0	0	4	17.4	1.000	0.046
AATY/KNTY	3	7.0	1	6.7	0	0	1.000	0.546
AATY/EYTY	3	7.0	1	6.7	0	0	1.000	0.546
AATY/ESTY	0	0	1	6.7	0	0	0.259	-
KNTY/KNTY	1	2.3	0	0	0	0	1.000	1.000
KNTY/EYTY	0	0	1	6.7	1	4.3	0.259	0.348

[a] Fisher's exact test was used to determine statistical significance

[b] Number of individual animals with a particular motif at position $\beta^{71\ 74\ 77\ and\ 78}$

Healthy	n=86	%	9	11	12	13	24	26	28	30	31	32	37	45	47	55	56	57	59	60	61	65	66	67	70	71	74	77	78	81	85	86
0101	4	4.7	E	S	K	S	V	F	L	D	Y	Y	T	T	G	F	R	Q	D	E	Y	W	D	F	E	K	E	R	V	H	G	M
14011	7	8.1	Q	H	-	G	-	L	-	-	H	F	Y	F	D	-	-	P	A	-	Q	-	-	-	Q	-	-	-	-	-	-	V
0701	7	8.1	-	C	-	R	-	-	-	-	C	F	H	F	-	-	-	R	V	-	Q	-	-	-	-	R	-	-	-	-	V	V
0902	5	5.8	-	-	-	-	-	-	-	E	S	F	Y	N	-	Y	-	P	-	-	-	-	E	I	-	R	-	-	-	-	V	G
2703	2	2.3	-	Y	N	-	-	-	D	C	-	T	-	G	F	R	P	D	E	Y	W	K	-	-	-	R	-	-	-	-	V	G
1101	6	7.0	Q	H	-	G	-	L	-	-	H	F	Y	Y	D	-	-	P	S	-	-	-	-	-	R	R	-	T	-	-	V	V
0501	1	1.2	-	H	-	-	L	Y	-	-	-	F	Y	Y	-	Y	-	P	-	K	-	-	E	I	R	-	N	T	Y	-	V	V
0502	1	1.2	-	H	-	-	L	Y	D	Y	F	-	V	G	Y	R	P	D	K	Y	W	K	-	L	R	-	N	T	Y	-	V	G
0503	3	3.5	-	-	-	-	L	Y	-	-	-	F	Y	Y	-	Y	-	P	-	K	-	-	E	I	R	-	N	T	Y	-	V	F
1302	7	8.1	-	L	-	-	-	-	-	E	S	F	Y	N	-	Y	-	P	-	K	-	-	-	L	R	-	N	T	Y	-	V	V
3401	1	1.2	-	C	-	-	-	-	-	E	S	F	Y	F	-	Y	-	R	V	-	Q	L	-	L	Q	-	N	T	Y	-	V	V
1601	13	15.1	-	T	-	K	-	-	-	-	-	F	H	F	-	Y	-	P	-	K	-	-	-	-	-	-	A	T	Y	-	V	G
20012	1	1.2	-	C	-	R	-	L	-	-	-	F	Y	R	-	-	-	P	S	-	-	-	-	-	Q	R	A	T	Y	-	V	V
0201	4	4.7	-	-	T	-	-	-	-	-	-	F	H	F	-	Y	-	P	-	-	-	-	E	I	R	A	A	T	Y	-	-	V
1001	10	11.6	-	-	-	-	-	-	-	-	-	F	H	Y	-	Y	Q	R	V	-	-	C	-	-	R	A	A	T	Y	-	V	G
1201	6	7.0	-	T	-	K	-	-	-	N	-	F	H	F	-	Y	-	P	-	-	-	-	E	I	R	A	A	T	Y	-	V	G
1501	8	9.3	-	-	T	-	-	Y	-	-	-	F	H	F	-	Y	-	R	V	-	Q	L	-	T	R	E	Y	T	Y	-	V	V
PL	n=30																															
0701	1	3.3	-	C	-	R	-	-	-	-	C	F	H	F	-	-	-	R	V	-	Q	-	-	-	-	R	-	-	-	-	V	V
1101	1	3.3	Q	H	-	G	-	L	-	-	H	F	Y	Y	D	-	-	P	S	-	-	-	-	-	R	R	-	T	-	-	V	V
0503	1	3.3	-	-	-	-	L	Y	-	-	-	F	Y	Y	-	Y	-	P	-	K	-	-	E	I	R	-	N	T	Y	-	V	F
1302	1	3.3	-	L	-	-	-	-	-	E	S	F	Y	N	-	Y	-	P	-	K	-	-	-	L	R	-	N	T	Y	-	V	V
m40sp6	1	3.3			-	-	L	Y	D	Y	F	-	Y	G	Y	R	P	D	K	Y	W	K	E	I	R	-	N	T	Y	-	V	F
1601	14	46.7	-	T	-	K	-	-	-	-	-	F	H	F	-	Y	-	P	-	K	-	-	-	-	-	-	A	T	Y	-	V	G
0201	1	3.3	-	-	T	-	-	-	-	-	-	F	H	F	-	Y	-	P	-	-	-	-	E	I	R	A	A	T	Y	-	-	V
1001	3	10.0	-	-	-	-	-	-	-	-	-	F	H	Y	-	Y	Q	R	V	-	-	C	-	-	R	A	A	T	Y	-	V	G
1201	1	3.3	-	T	-	K	-	-	-	N	-	F	H	F	-	Y	-	P	-	-	-	-	E	I	R	A	A	T	Y	-	V	G
1501	5	16.7	-	-	T	-	-	Y	-	-	-	F	H	F	-	Y	-	R	V	-	Q	L	-	T	R	E	Y	T	Y	-	V	V
0801	1	3.3	-	A	T	-	-	-	D	Y	F	H	L	G	F	R	P	S	V	H	L	K	-	-	D	E	S	T	Y	-	V	V
Lymphoma	n=46																															
0902	2	4.3	-	-	-	-	-	-	-	E	S	F	Y	N	-	Y	-	P	-	-	-	-	E	I	-	R	-	-	-	-	V	G
1101	3	6.5	Q	H	-	G	-	L	-	-	H	F	Y	Y	D	-	-	P	S	-	-	-	-	-	R	R	-	T	-	-	V	V
0501	1	2.2	-	H	-	-	L	Y	-	-	-	F	Y	Y	-	Y	-	P	-	K	-	-	E	I	R	-	N	T	Y	-	V	V
3401	1	2.2	-	C	-	-	-	-	-	E	S	F	Y	F	-	Y	-	R	V	-	Q	L	-	L	Q	-	N	T	Y	-	V	V
1601	16	34.8	-	T	-	K	-	-	-	-	-	F	H	F	-	Y	-	P	-	K	-	-	-	-	-	-	A	T	Y	-	V	G
20012	3	6.5	-	C	-	R	-	L	-	-	-	F	Y	R	-	-	-	P	S	-	-	-	-	-	Q	R	A	T	Y	-	V	V
0201	5	10.9	-	-	T	-	-	-	-	-	-	F	H	F	-	Y	-	P	-	-	-	-	E	I	R	A	A	T	Y	-	-	V
1001	11	23.9	-	-	-	-	-	-	-	-	-	F	H	Y	-	Y	Q	R	V	-	-	C	-	-	R	A	A	T	Y	-	V	G
1201	1	2.2	-	T	-	K	-	-	-	N	-	F	H	F	-	Y	-	P	-	-	-	-	E	I	R	A	A	T	Y	-	V	G
4401	1	2.2	-	Y	-	-	-	-	D	Y	-	T	-	G	F	R	Q	D	E	Q	W	K	-	-	R	A	A	T	Y	Y	V	G
1501	2	4.3	-	-	T	-	-	Y	-	-	-	F	H	F	-	Y	-	R	V	-	Q	L	-	T	R	E	Y	T	Y	-	V	V

Fig. 4-1. Alignment of variable codon of *BoLA-DRB3*alleles according to each stage such as Healthy, PL and Lymphosarcoma. Distinctive motif were squared.

SUMMARY

The bovine leukocyte antigen (*BoLA*) class II genes code for highly polymorphic transmembrane glycoproteins that present antigenic peptides to helper T cells. The *BoLA-DRB3* is thought to be the most functionally significant as it is actively transcribed and in excess of 102 alleles have been characterized for this locus. First, we developed the technique of polymerase chain reaction-sequence based typing (PCR-SBT) which can assign *DRB3* alleles in DNA sequence level and would allow fast screening of large numbers of animals. Next, we discuss the relationship between the results of PCR-SBT typing and the identification of *BoLA-DRB3* alleles by PCR-RFLP analysis for check this method. And then, we designed to determine the nucleotide sequences of *exon 2* of *BoLA-DRB3* alleles in a herd of 29 Japanese Shorthorn cattle of experimental farm, graduate school of agricultural science, Tohoku University, using PCR-SBT and to compare the results with *BoLA-DRB3* haplotypes, as determined by PCR-RFLP. The result also shows that the PCR-SBT method was useful for field samples. Third, we designed to determine the nucleotide sequences of *exon 2* of *BoLA-DRB3* alleles in a total of 419 animals from four distinct cattle breeds such as Japanese Black, Japanese Shorthorn, Holstein and Jersey by our PCR-SBT method, and the allele frequencies and the phylogenetic relationship of *BoLA-DRB3* alleles and the genetic background of these populations are discussed. Finally, clinical application of PCR-SBT method was described.

1. *Development of BoLA-DRB3 PCR-SBT method*

In this study, we developed a new method for sequence based typing (SBT) of alleles of this locus that appears to be generally applicable to all BoLA class II genes. First, we performed an initial round of amplification by PCR using conserved locus-specific primers. Then we performed PCR with individual sequence-specific primers (SSPs). For this second round of amplification, we used a locus-specific primer and at least one group-specific primers to amplify all the alleles that belonged to eight groups. These eight groups of alleles were categorized in terms of the sequence of amino acids 9-13 in the first hypervariable region of BoLA-DRβ. The locus-specific primer DRB3ALL was also used for the second PCR to avoid to skipping a new allele or one of the recognized alleles. Next, we performed cycle sequencing of each product using an M13-tailed system.

2. *Verification of PCR-SBTmethod*

To verify this method, we investigated 52 animals whose *BoLA-DRB3* haplotypes had been characterized at the Fifth International BoLA Workshop. I identified 27 different alleles that included four new alleles in 43 different heterozygous and two different homozygous combinations. The reproducibility was 100% for all samples on two separate occasions. The results of PCR-SBT exactly matched the PCR-RFLP patterns as defined at the Fifth International BoLA Workshop. Collectively, the results suggest that our method allows detection of as yet undefined alleles that consist of new combinations of known sequences. Next, we try to apply this method to field samples. This study was made of *exon 2* in the *BoLA-DRB3* of 29 Japanese Shorthorn cattle at the Kawatabi Farm of the University of Tohoku using polymerase chain reaction-sequence-based

typing (PCR-SBT). I performed an initial round of PCR using conserved locus-specific primers and a second round of PCR using a locus-specific primer and at least one group-specific primer. I found that the *BoLA-DRB3* alleles of the 29 individuals could be clearly divided into seven independent groups (G1, G2, G4 through G8). Direct sequencing of products of PCR with sequence-specific primers (SSP) revealed that these individuals had nine distinct previously published alleles: *BoLA-DRB3*0101, *0201, *0301, *0501, *0801, *0901, *1101, *1201* and **14011*. The *DRB3*1201* allele was found at the highest frequency (24.1%) and *DRB3*14011* was the second most frequent allele (20.7%). I digested the products of amplification by PCR of *BoLA-DRB3 exon 2* with *Rsa*I, *BstY*I and *Hae*III and identified nine previously described PCR-RFLP patterns (*BoLA-DRB3.2*01, *07, *08, *09, *11, *21, *22, *24* and **27*). The PCR-RFLP patterns reflected the results of PCR-SBT exactly. Our results indicate that *exon 2* of the *BoLA-DRB3* gene is highly polymorphic in Japanese Shorthorn cattle and that PCR-SBT can improve the resolution and the accuracy of typing of *BoLA-DRB3* alleles in large numbers of animals.

3. Characterization of BoLA-DRB3 alleles in four distinct cattle breed

The genotypes of 419 samples from four different cattle populations from Japan were determined for *exon 2* of the *BoLA-DRB3* allele by PCR-SBT. The 35 different previously published alleles and the three novel alleles were identified: 20 published alleles from 102 Japanese Shorthorn; 12 published alleles from 17 Jersey; 18 published alleles from 100 Holstein; and 24 alleles including 3 new alleles from 200 Japanese Black. To differentiate the allelic variations between four distinct breeds, the gene frequencies of the *BoLA-DRB3* alleles in each breed were compared with those of other populations. All breeds examined were found to carry extremely

high *DRB3* diversities, with heterozygosity rates of between 90.5% and 100%, and, in addition, the remarkably dissimilar distribution patterns of *BoLA-DRB3* alleles between other breeds. Moreover, it appeared that the *BoLA-DRB3* sequences isolated from single breed were shared between other breeds. This result confirmed by a phylogenetic tree constructed from *DRB3* nucleotide sequences. Moreover, the phylogenetic tree constructed based on the allele frequencies of the *BoLA-DRB3* in these breeds using the Neighbour-joining method suggested that Holstein and Japanese Black were the closest to each other, but Jersey was farther from the these both breeds than Japanese Shorthorn.

4. *Bovine Leukemia Virus-associated Leukemia was defined by BoLA-DRB3genes*

The nucleotide sequencing of *exon 2* of the *BoLA-DRB3* gene, which is highly polymorphic, of 81 BLV-infected animals with 3 independent stages such as an aleukemic healthy, persistent lymphocytosis (PL) and lymphoma was determined by PCR-sequenced-based typing. I identified 23 distinct *BoLA-DRB3* alleles, including 3 new alleles. The population of healthy cattle positive for *BoLA-DRB3*14011* was higher than the proportion-bearing individuals in 200 control cases that were positive for the same allele. By contrast, the *BoLA-DRB3*1601* alleles was found most frequently in cattle with PL and lymphoma. Sequence analysis revealed that, approximately 56% of 43 BLV-infected but healthy cattle carry at least one *BoLA-DRB3* allele encoding Arg^{71} or Lys^{71}, Glu^{74}, Arg^{77} and Val^{78} of β1 domain of DR molecule, which suggested that alleles encoding the KERV and RERV motifs might protect against tumor development. By contrast, approximately 70% of 23 BLV-infected cattle with lymphoma carry two alleles encoding Ala^{74}, Thr^{77} and Tyr^{78}, indicating that the ATY/ ATY genotype might be associated with

susceptibility to lymphoma. Such, these results suggest that the existence of alleles associated with resistance and susceptibility to BLV-induced leukemogenesis.

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References

Ababou A, Goyeneche J, Davis WC, Levy D (1994) Evidence for the expression of three different BoLA-class II molecules on the bovine BL-3 cell line: determination of a non-DR non-DQ gene product. J Leukoc Biol 56:182-186.

Aida Y (1995) Characterization and expression of bovine MHC class II genes. Bull. Soc. Fr. Jpn. Sci. Vet. 6: 17-24

Aida Y, Okada K, Amanuma H (1993) Phenotype and ontogeny of cells carrying a tumor-associated antigen that is expressed on bovine leukemia virus-induced lymphosarcoma. Cancer Res 53:429-437.

Aida Y, Okada K, Ohtsuka M, Amanuma H (1992) Tumor-associated M(r) 34,000 and M(r) 32,000 membrane glycoproteins that are serine phosphorylated specifically in bovine leukemia virus- induced lymphosarcoma cells. Cancer Res 52:6463-6470.

Aida Y, Niimi M, Asahina M, Okada K, Nakai Y, Ogimoto K (1995) Identification of a new bovine MHC class II DRB allele by nucleotide sequencing and an analysis of phylogenetic relationships. Biochem Biophys Res Commun 209:981-988.

Aldridge BM, McGuirk SM, Clark RJ, Knapp LA, Watkins DI, Lunn DP (1998) Denaturing gradient gel electrophoresis: a rapid method for differentiating BoLA-DRB3 alleles. Anim Genet 29:389-394.

Ammer H, Schwaiger FW, Kammerbauer C, Gomolka M, Arriens A, Lazary S, Epplen JT (1992) Exonic polymorphism vs intronic simple repeat hypervariability in MHC- DRB genes.

Immunogenetics 35:332-340.

Amorena B, Stone WH (1978) Serologically defined (SD) locus in cattle. Science 201:159-160.

Andersson L, Bohme J, Peterson PA, Rask L (1986b) Genomic hybridization of bovine class II major histocompatibility genes: 2. Polymorphism of DR genes and linkage disequilibrium in the DQ- DR region. Anim Genet 17:295-304.

Andersson L, Bohme J, Rask L, Peterson PA (1986a) Genomic hybridization of bovine class II major histocompatibility genes: 1. Extensive polymorphism of DQ alpha and DQ beta genes. Anim Genet 17:95-112.

Andersson L, Lunden A, Sigurdardottir S, Davies CJ, Rask L (1988) Linkage relationships in the bovine MHC region. High recombination frequency between class II subregions. Immunogenetics 27:273-280.

Band M, Larson JH, Womack JE, Lewin HA (1998) A radiation hybrid map of BTA23: identification of a chromosomal rearrangement leading to separation of the cattle MHC class II subregions. Genomics 53:269-275.

Bensaid A, Kaushal A, Baldwin CL, Clevers H, Young JR, Kemp SJ, MacHugh ND, Toye PG, Teale AJ (1991) Identification of expressed bovine class I MHC genes at two loci and demonstration of physical linkage. Immunogenetics 33:247-254.

Bissumbhar B, Nilsson PR, Hensen EJ, Davis WC, Joosten I (1994) Biochemical characterization of bovine MHC DQ allelic variants by one- dimensional isoelectric focusing. Tissue Antigens 44:100-109.

Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC (1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens.

Nature 329:512-518.

Bodmer WF (1972) Evolutionary significance of the HL-A system. Nature 237:139-145 passim.

Brown JH, Jardetzky T, Saper MA, Samraoui B, Bjorkman PJ, Wiley DC (1988) A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules [published erratum appears in Nature 1988 Jun 23;333(6175):786]. Nature 332:845-850.

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364:33-39.

Burke MG, Stone RT, Muggli-Cockett NE (1991) Nucleotide sequence and northern analysis of a bovine major histocompatibility class II DR beta-like cDNA. Anim Genet 22:343-352.

Burny A, Cleuter Y, Kettmann R, Mammerickx M, Marbaix G, Portetelle D, Van den Broeke A, Willems L, Thomas R (1988) Bovine leukemia: facts and hypotheses derived from the study of an infectious cancer. Adv Vet Sci Comp Med 32:149-170.

Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, Strominger JL (1992) Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature 358:764-768.

Coffin JM (1996) Retroviridae: the viruses and their replication. In: B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L., Melnick, T. P. Monath, B. Roizman, and S. E. Straus (eds.), Fields Virology, 2nd ed., Vol. 2. pp. 1767-1847. Philadelphia: Lippincott-Raven

Cresswell P (1994) Assembly, transport, and function of MHC class II molecules. Annu Rev Immunol 12:259-293.

Davies CJ, Antczak DF (1991a) Mixed lymphocyte culture studies reveal complexity in the bovine

MHC not detected by class I serology. Anim Genet 22:31-44.

Davies CJ, Antczak DF (1991b) Production and characterization of alloantisera specific for bovine class II major histocompatibility complex antigens. Anim Genet 22:417-434.

Davies CJ, Andersson L, Joosten I, Mariani P, Gasbarre LC, Hensen EJ (1992) Characterization of bovine MHC class II polymorphism using three typing methods: serology, RFLP and IEF. Eur J Immunogenet 19:253-262.

Davies CJ, Joosten I, Andersson L, Arriens MA, Bernoco D, Bissumbhar B, Byrns G, van Eijk MJ, Kristensen B, Lewin HA, et al. (1994a) Polymorphism of bovine MHC class II genes. Joint report of the Fifth International Bovine Lymphocyte Antigen (BoLA) Workshop, Interlaken, Switzerland, 1 August 1992. Eur J Immunogenet 21:259-289.

Davies CJ, Joosten I, Bernoco D, Arriens MA, Bester J, Ceriotti G, Ellis S, Hensen EJ, Hines HC, Horin P, et al. (1994b) Polymorphism of bovine MHC class I genes. Joint report of the Fifth International Bovine Lymphocyte Antigen (BoLA) Workshop, Interlaken, Switzerland, 1 August 1992. Eur J Immunogenet 21:239-258.

Davis WC, Marusic S, Lewin HA, Splitter GA, Perryman LE, McGuire TC, Gorham JR (1987) The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. Vet Immunol Immunopathol 15:337-376.

Derse D (1988) trans-acting regulation of bovine leukemia virus mRNA processing. J Virol 62:1115-1119.

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for

the VAX. Nucleic Acids Res 12:387-395.

Dietz AB, Cohen ND, Timms L, Kehrli ME, Jr. (1997a) Bovine lymphocyte antigen class II alleles as risk factors for high somatic cell counts in milk of lactating dairy cows. J Dairy Sci 80:406-412.

Dietz AB, Detilleux JC, Freeman AE, Kelley DH, Stabel JR, Kehrli ME, Jr. (1997b) Genetic association of bovine lymphocyte antigen DRB3 alleles with immunological traits of Holstein cattle. J Dairy Sci 80:400-405.

Dikiniene N, Aida Y (1995) Cattle cDNA clones encoding MHC class II DQB1 and DQB2 genes. Immunogenetics 42:75.

Djilali S, Parodi AL, Levy D, Cockerell GL (1987) Development of leukemia and lymphosarcoma induced by bovine leukemia virus in sheep: a hematopathological study. Leukemia 1:777-781.

Doherty PC, Zinkernagel RM (1975) Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. Nature 256:50-52.

Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12:241-253.

Ellegren H, Davies CJ, Andersson L (1993) Strong association between polymorphisms in an intronic microsatellite and in the coding sequence of the BoLA-DRB3 gene: implications for microsatellite stability and PCR-based DRB3 typing. Anim Genet 24:269-275.

Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351:290-296.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Fitzpatrick JL, Cripps PJ, Hill AW, Bland PW, Stokes CR (1992) MHC class II expression in the bovine mammary gland. Vet Immunol Immunopathol 32:13-23.

French MH, Johansson I, Joshi NR, McLaughlin EA (1996) European Breeds of Cattle. Food and Agricultural Organization of the United Nations, Rome.

Gelhaus A, Schnittger L, Mehlitz D, Horstmann RD, Meyer CG (1995) Sequence and PCR-RFLP analysis of 14 novel BoLA-DRB3 alleles. Anim Genet 26:147-153.

Germain RN, Margulies DH (1993) The biochemistry and cell biology of antigen processing and presentation. Annu Rev Immunol 11:403-450.

Gilliespie BE, Jayarao BM, Dowlen HH, Oliver SP (1999) Analysis and frequency of bovine lymphocyte antigen DRB3.2 alleles in Jersey cows. J Dairy Sci 82:2049-2053.

Giovambattista G, Golijow CD, Dulout FN, Lojo MM (1996) Gene frequencies of DRB3.2 locus of Argentine Creole cattle. Anim Genet 27:55-56.

Gish W, States DJ (1993) Identification of protein coding regions by database similarity search. Nat Genet 3:266-272.

Glass EJ, Oliver RA, Williams JL, Millar P (1992) Alloreactive T-cell recognition of bovine major histocompatibility complex class II products defined by one-dimensional isoelectric focusing. Anim Genet 23:97-111.

Groenen MA, van der Poel JJ, Dijkhof RJ, Giphart MJ (1990) The nucleotide sequence of bovine MHC class II DQB and DRB genes [published errata appear in Immunogenetics 1992;35(4):290 and 1993;37(4):315]. Immunogenetics 31:37-44.

Hidaka M, Inoue J, Yoshida M, Seiki M (1988) Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins. EMBO J 7: 519-523.

Hoang-Xuan M, Charron D, Zilber MT, Levy D (1982) Biochemical characterization of class II bovine major histocompatibility complex antigens using cross-species reactive antibodies. Immunogenetics 15:621-624.

Hughes AL, Nei M (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature 335:167-170.

Hughes AL, Nei M (1989) Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. Proc Natl Acad Sci U S A 86:958-962.

Hughes HP, Campos M, McDougall L, Beskorwayne TK, Potter AA, Babiuk LA (1994) Regulation of major histocompatibility complex class II expression by Pasteurella haemolytica leukotoxin. Infect Immun 62:1609-1615.

Hughes SH, Shank PR, Spector DH, Kung HJ, Bishop JM, Varmus HE, Vogt PK, Breitman ML (1978) Proviruses of avian sarcoma virus are terminally redundant, co- extensive with unintegrated linear DNA and integrated at many sites. Cell 15:1397-1410.

Janzer-Pfeil AM, Splitter GA (1989) Identification of two independent MHC class II antigens in a bovine lymphoblastoid cell line. Vet Immunol Immunopathol 22:307-319.

Joosten I, Sanders MF, van der Poel A, Williams JL, Hepkema BG, Hensen EJ (1989) Biochemically defined polymorphism of bovine MHC class II antigens. Immunogenetics 29:213-216.

Jorgensen JL, Esser U, Fazekas de St. Groth B, Reay PA, Davis MM (1992) Mapping T-cell

receptor-peptide contacts by variant peptide immunization of single-chain transgenics. Nature 355:224-230.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111-120.

Klein J (1987) Origin of major histocompatibility complex polymorphism: the trans- species hypothesis. Hum Immunol 19:155-162.

Klein J, Bontrop RE, Dawkins RL, Erlich HA, Gyllensten UB, Heise ER, Jones PP, Parham P, Wakeland EK, Watkins DI (1990) Nomenclature for the major histocompatibility complexes of different species: a proposal. Immunogenetics 31:217-219.

Klein J, Satta Y, O'Huigin C, Takahata N (1993) The molecular descent of the major histocompatibility complex. Annu Rev Immunol 11:269-295.

Knipper AJ, Hinney A, Schuch B, Enczmann J, Uhrberg M, Wernet P (1994) Selection of unrelated bone marrow donors by PCR-SSP typing and subsequent nonradioactive sequence-based typing for HLA DRB1/3/4/5, DQB1, and DPB1 alleles. Tissue Antigens 44:275-284.

Kotsch K, Wehling J, Blasczyk R (1999) Sequencing of HLA class II genes based on the conserved diversity of the non-coding regions: sequencing based typing of HLA-DRB genes. Tissue Antigens 53:486-497.

Kumar S, Tamura K, Nei M (1993) MEGA: molecular evolutionary genetic analysis, version 1.01, The Pennsylvania State University, University Park, Pa

Lalor PA, Morrison WI, Goddeeris BM, Jack RM, Black SJ (1986) Monoclonal antibodies identify phenotypically and functionally distinct cell types in the bovine lymphoid system. Vet Immunol Immunopathol 13:121-140.

Lessa EP (1993) Analysis of DNA sequence variation at population level by polymerase chain reaction and denaturing gradient gel electrophoresis. In: Zimmer EA, White TJ, Cann RL, Wilson AC (eds): Methods in Enzymology Volume 224, Molecular evolution: Producing the Biochemical Data. Academic Press Inc., California, pp 419-428.

Levy D, Deshayes L, Guillemain B, Parodi AL (1977) Bovine leukemia virus specific antibodies among French cattle. I. Comparison of complement fixation and hematological tests. Int J Cancer 19:822-827.

Lewin HA (1996) Genetic organization, polymorphism, and function of the bovine major histocompatibility complex. In: Schook LB, Lamont SJ (eds): CRC Series in Comparative Immunology, The Major Histocompatibility Complex Region of Domestic Animal Species, CRC Press, Florida, pp 65-98.

Lewin HA, Davis WC, Bernoco D (1985a) Monoclonal antibodies that distinguish bovine T and B lymphocytes. Vet Immunol Immunopathol 9:87-102.

Lewin HA, Calvert CC, Bernoco D (1985b) Cross-reactivity of a monoclonal antibody with bovine, equine, ovine, and porcine peripheral blood B lymphocytes. Am J Vet Res 46:785-788.

Maillard JC, Renard C, Chardon P, Chantal I, Bensaid A (1999) Characterization of 18 new BoLA-DRB3 alleles. Anim Genet 30:200-203.

Manns A, Hanchard B, Morgan OS, Wilks R, Cranston B, Nam JM, Blank M, Kuwayama M, Yashiki S, Fujiyoshi T, Blattner W, Sonoda S (1998) Human leukocyte antigen class II alleles associated with human T-cell lymphotropic virus type I infection and adult T-cell leukemia/lymphoma in a Black population. J Natl Cancer Inst 90:617-622.

McGinnis MD, Conrad MP, Bouwens AG, Tilanus MG, Kronick MN (1995) Automated, solid-

phase sequencing of DRB region genes using T7 sequencing chemistry and dye-labeled primers. Tissue Antigens 46:173-179.

McKnight GS (1978) The induction of ovalbumin and conalbumin mRNA by estrogen and progesterone in chick oviduct explant cultures. Cell 14:403-413.

Mikko S, Andersson L (1995a) Low major histocompatibility complex class II diversity in European and North American moose. Proc Natl Acad Sci U S A 92:4259-4263.

Mikko S, Andersson L (1995b) Extensive MHC class II DRB3 diversity in African and European cattle. Immunogenetics 42:408-413.

Muggli-Cockett NE, Stone RT (1988) Identification of genetic variation in the bovine major histocompatibility complex DR beta-like genes using sequenced bovine genomic probes. Anim Genet 19:213-225.

Muggli-Cockett NE, Stone RT (1989) Partial nucleotide sequence of a bovine major histocompatibility class II DR beta-like gene. Anim Genet 20:361-369.

Murray BW, White BN (1998) Sequence variation at the major histocompatibility complex DRB loci in beluga (Delphinapterus leucas) and narwhal (Monodon monoceros). Immunogenetics 48:242-252.

Myers RM, Sheffield VC, Cox DR (1988) Detection of single base changes in DNA: ribonuclease cleavage and denaturing gradient gel electrophoresis. In: Davies KE (ed): Genome Analysis, a Practical Approach, IRL Press Inc., Washington, DC, pp95-139.

Nagaoka Y, Kabeya H, Onuma M, Kasai N, Okada K, Aida Y (1999) Ovine MHC class II DRB1 alleles associated with resistance or susceptibility to development of bovine leukemia virus-induced ovine lymphoma. Cancer Res 59:975-981.

Namikawa T (1980) Genetical aspect of domestication and phylogeny in the cattle. Jap J Zoothech Sci 49: 817-827.

Nei M (1972) Genetic distance between populations. American Nature 106: 283-292.

Nishino Y, Tajima S, Aida Y (1995) Cattle cDNA clone encoding a new allele of the MHC class II DQA1 gene. Immunogenetics 42:306-307.

Ogawa Y, Daigo M, Amasaki H (1989) Craniometrical estimation of the native Japanse Mishima cattle. Anat Anz Jena 168: 197-203.

Paabo S, Higuchi RG, Wilson AC (1989) Ancient DNA and the polymerase chain reaction. The emerging field of molecular archaeology. J Biol Chem 264:9709-9712.

Rothel JS, Dufty JH, Wood PR (1990) Studies on the bovine major histocompatibility class I and class II antigens using homozygous typing cells and antigen-specific BoT4+ blast cells. Anim Genet 21:141-148.

Rudensky A, Preston-Hurlburt P, al-Ramadi BK, Rothbard J, Janeway CA, Jr. (1992) Truncation variants of peptides isolated from MHC class II molecules suggest sequence motifs. Nature 359:429-431.

Rukstalis DB, Bubley GJ, Donahue JP, Richie JP, Seidman JG, DeWolf WC (1989) Regional loss of chromosome 6 in two urological malignancies. Cancer Res 49:5087-5090.

Russell GC, Davies CJ, Andersson L, Ellis SA, Hensen EJ, Lewin HA, Mikko S, Muggli-Cockett NE, van der Poel JJ (1997) BoLA class II nucleotide sequences, 1996: Report of the ISAG BoLA Nomenclature Committee. Anim Genet 28: 169-180.

Russell GC, Fraser DC, Craigmile S, Oliver RA, Dutia BM, Glass EJ (2000) Sequence and transfection of *BoLA-DRB3* cDNAs. Anim Genet 31:219-222.

Russell GC, Davies CJ, Ellis S, Hensen EJ, Lewin HA, Skow L ISAG BoLA Nomenclature Committee Web Site.: http://www.ri.bbsrc.ac.uk/bola/bolahome.htm

Sagata N, Tsuzuku-Kawamura J, Nagayoshi-Aida M, Shimizu F, Imagawa K, Ikawa Y (1985) Identification and some biochemical properties of the major XBL gene product of bovine leukemia virus. Proc Natl Acad Sci U S A 82:7879-7883.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463-5467.

Santamaria P, Boyce-Jacino MT, Lindstrom AL, Barbosa JJ, Faras AJ, Rich SS (1992) HLA class II "typing": direct sequencing of DRB, DQB, and DQA genes. Hum Immunol 33:69-81.

Scheltinga SA, Williams F, van der Zwan AW, Rozemuller EH, Middleton D, Tilanus MG (1998) HLA-A towards a high-resolution DNA typing. Tissue Antigens 51:549-552.

Seiki M, Inoue J, Hidaka M, Yoshida M (1988) Two cis-acting elements responsible for posttranscriptional trans- regulation of gene expression of human T-cell leukemia virus type I. Proc Natl Acad Sci U S A 85:7124-7128.

Sharif S, Mallard BA, Wilkie BN, Sargeant JM, Scott HM, Dekkers JC, Leslie KE (1998) Associations of the bovine major histocompatibility complex DRB3 (BoLA- DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Anim Genet 29:185-193.

Sigurdardottir S, Lunden A, Andersson L (1988) Restriction fragment length polymorphism of DQ and DR class II genes of the bovine major histocompatibility complex. Anim Genet

19:133-150.

Sigurdardottir S, Borsch C, Gustafsson K, Andersson L (1991) Cloning and sequence analysis of 14 DRB alleles of the bovine major histocompatibility complex by using the polymerase chain reaction. Anim Genet 22:199-209.

Sitte K, East IJ, Jazwinska EC (1996) Detection of a common BoLA-DRB3 deletion by sequence-specific oligonucleotide typing. Anim Genet 27:271-273.

Sitte K, East IJ, Lavin MF, Jazwinska EC (1995) Identification and characterization of new BoLA-DRB3 alleles by heteroduplex analysis and direct sequencing. Anim Genet 26:413-417.

Slierendregt BL, Otting N, Kenter M, Bontrop RE (1995) Allelic diversity at the Mhc-DP locus in rhesus macaques (Macaca mulatta). Immunogenetics 41:29-37.

Sodroski JG, Rosen CA, Haseltine WA (1984) Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science 225:381-385.

Spooner RL, Leveziel H, Grosclaude F, Oliver RA, Vaiman M (1978) Evidence for a possible major histocompatibility complex (BLA) in cattle. J Immunogenet 5:325-346.

Spooner RL, Oliver RA, Sales DI, McCoubrey CM, Millar P, Morgan AG, Amorena B, Bailey E, Bernoco D, Brandon M, Bull RW, Caldwell J, Cwik S, van Dam RH, Dodd J, Gahne B, Grosclaude F, Hall JG, Hines H, Leveziel H, Newman MJ, Stear MJ, Stone WH, Vaiman M (1979) Analysis of alloantisera against bovine lymphocytes. Joint report of the 1st International Bovine Lymphocyte Antigen (BoLA) workshop. Anim Blood Groups Biochem Genet 10:63-86.

Spurzem JR, Sacco O, Rossi GA, Beckmann JD, Rennard SI (1992) Regulation of major histocompatibility complex class II gene expression on bovine bronchial epithelial cells. J.

Lab. Clin. Med. 120: 94.

Spurkland A, Knutsen I, Markussen G, Vartdal F, Egeland T, Thorsby E (1993) HLA matching of unrelated bone marrow transplant pairs: direct sequencing of in vitro amplified HLA-DRB1 and -DQB1 genes using magnetic beads as solid support. Tissue Antigens 41:155-164.

Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, Wiley DC (1994) Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature 368:215-221.

Stone RT, Muggli-Cockett NE (1993) BoLA-DIB: species distribution, linkage with DOB, and northern analysis. Anim Genet 24:41-45.

Swarbrick PA, Schwaiger FW, Epplen JT, Buchan GS, Griffin JF, Crawford AM (1995) Cloning and sequencing of expressed DRB genes of the red deer (Cervus elaphus) Mhc. Immunogenetics 42:1-9.

Takeshima S, Ikegami M, Morita M, Nakai Y, Aida Y (2000) Identification of new bovine BoLA-DRB3 alleles by sequence-based typing. Immunogenetics: in press.

Tajima S, Zhuang WZ, Kato MV, Okada K, Ikawa Y, Aida Y (1998) Function and Conformation of Wild-Type p53 Protein Are Influenced by Mutations in Bovine Leukemia Virus-Induced B cell Lymphosarcoma. Virology 243:235-246.

Taylor BC, Choi KY, Scibienski RJ, Moore PF, Stott JL (1993) Differential expression of bovine MHC class II antigens identified by monoclonal antibodies. J Leukoc Biol 53:479-489.

Van Den Bussche RA, Hoofer SR, Lochmiller RL (1999) Characterization of Mhc-DRB allelic diversity in white-tailed deer (Odocoileus virginianus) provides insight into Mhc-DRB

allelic evolution within Cervidae. Immunogenetics 49:429-437.

van Eijk MJ, Stewart-Haynes JA, Lewin HA (1992) Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. Anim Genet 23:483-496.

van Eijk MJ, Beever JE, Da Y, Stewart JA, Nicholaides GE, Green CA, Lewin HA (1995) Genetic mapping of BoLA-A, CYP21, DRB3, DYA, and PRL on BTA23. Mamm Genome 6:151-152.

Voorter CE, de Bruyn-Geraets D, van den Berg-Loonen EM (1997) High-resolution HLA typing for the DRB3/4/5 genes by sequence-based typing. Tissue Antigens 50:283-290.

Watkins DI, Shadduck JA, Rudd CE, Stone ME, Lewin HA, Letvin NL (1989) Isoelectric focusing of bovine major histocompatibility complex class II molecules. Eur J Immunol 19:567-570.

Weber CK, Shaffer DJ, Sidman CL (1991) Unexpected behavior of H2Kb mutant DNAs in denaturing gradient gel electrophoresis. Nucleic Acids Res 19:3331-3335.

Xu A, McKenna K, Lewin HA (1993a) Sequencing and genetic analysis of a bovine DQA cDNA clone. Immunogenetics 37:231-234.

Xu A, Park C, Lewin HA (1994) Both DQB genes are expressed in BoLA haplotypes carrying a duplicated DQ region. Immunogenetics 39:316-321.

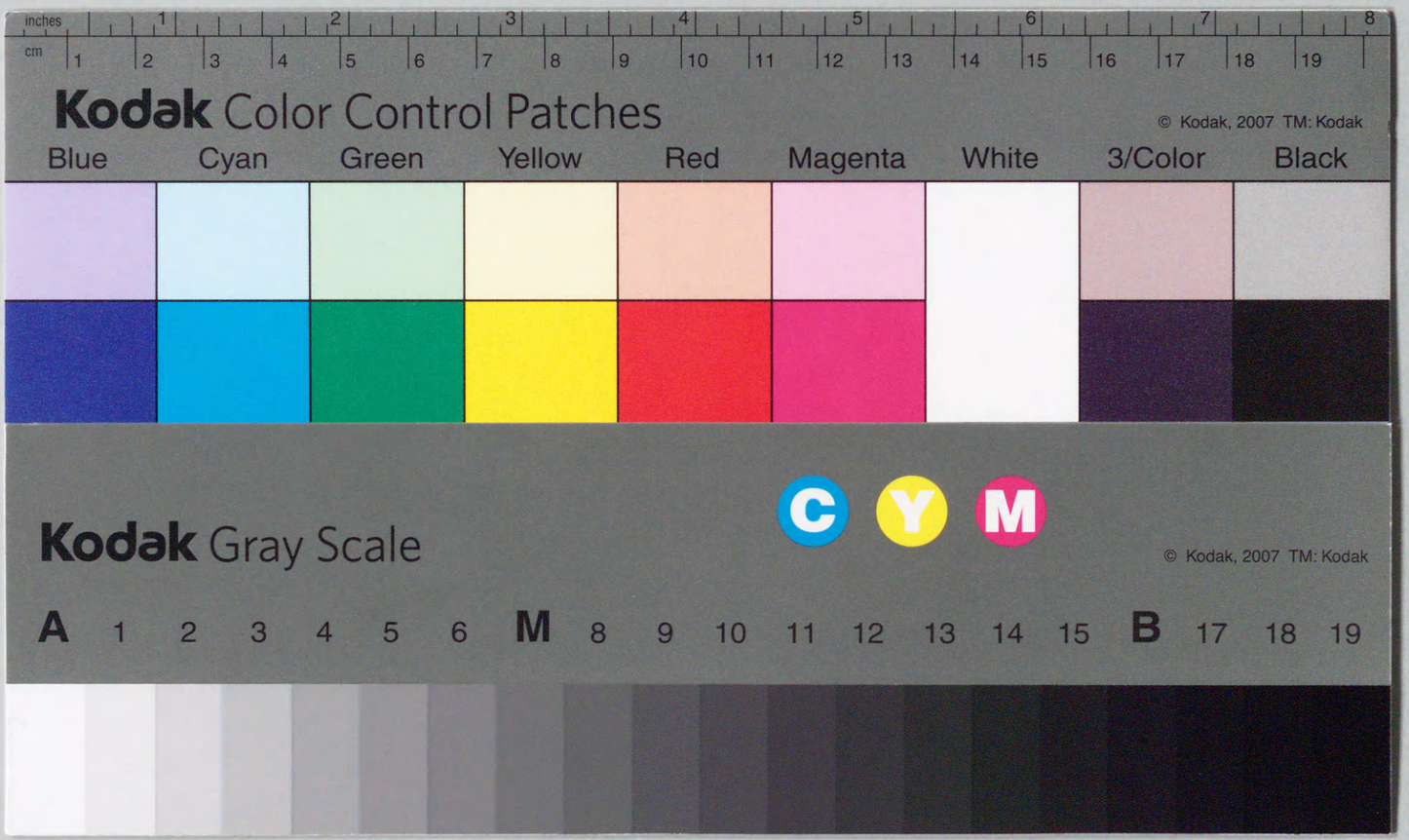
Xu A, van Eijk MJ, Park C, Lewin HA (1993b) Polymorphism in BoLA-DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. J Immunol 151:6977-6985.

Xu A, Clark TJ, Teutsch MR, Schook LB, Lewin HA (1991) Sequencing and genetic analysis of a bovine DQB cDNA clone. Anim Genet 22:381-398.

Yuhki N, O'Brien SJ (1997) Nature and origin of polymorphism in feline MHC class II DRA and DRB genes. J Immunol 158:2822-2833.

Zanotti M, Poli G, Ponti W, Polli M, Rocchi M, Bolzani E, Longeri M, Russo S, Lewin HA, van Eijk MJ (1996) Association of BoLA class II haplotypes with subclinical progression of bovine leukaemia virus infection in Holstein-Friesian cattle. Anim Genet 27:337-341.

Development of TCR-sequence based typing (SBT) method and its application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